CYTOKININ-INDUCED GENE EXPRESSION IN *ARABIDOPSIS*

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ABSTRACT

Cytokinins are plant hormones that affect the primary growth of shoots and roots. Application of the cytokinin N^6-benzylaminopurine (BAP) to the shoot apical meristem of *Arabidopsis thaliana* Landsberg *erecta* (L.) Heynh induces aberrant flower development and a significant genetic response, and some of these phenotypes and expression patterns were carried to the next generation. Analysis of altered transcript levels with Affymetrix GeneChips® indicated significant changes in transcript levels of genes associated with shoot meristem activity, circadian rhythms, cytokinin metabolism, two-component systems, stress and defense responses, auxin regulation, ethylene and salicylic acid biosynthesis, and signal transduction. Specific genes were also mined from the data as potentially responsible for the BAP-induced aberrant floral phenotypes, increased floral organ number, buds in axils of sepals, and mosaic floral organs. Of particular note was a decrease in the transcript levels of *CLAVATA1* (*CLV1*), a gene encoding a receptor kinase involved in organ differentiation and maintenance of shoot and floral meristems. Time course analysis by RT-PCR showed a decline and subsequent recovery of transcript levels of *CLV1* and a coincident increase in *WUSCHEL* (*WUS*) transcript, consistent with the known suppression of *WUS* by CLV. *WUS* encodes a homeodomain protein associated with shoot meristem proliferation. The temporal coincidence of an increased floral organ phenotype with changes in transcript levels of *CLV1* and *WUS* suggests that cytokinins regulate flower development by affecting the activity of genes controlling shoot meristem activity. Aberrant floral phenotypes in subsequent non-treated generations suggest epigenetic inheritance of some BAP-altered transcript patterns. Repressed expression of the majority of significant genes in the untreated T_1 population suggests a mechanism of gene silencing, such as methylation, was involved in this epigenetic inheritance. Also, transcript levels of time-keeping genes, including *CIRCADIAN CLOCK ASSOCIATED 1* / *ELONGATED HYPOCOTYL*, and associated genes with oscillating expression patterns, such as *COLD-RESPONSIVE*, were affected by BAP in treated plants and the subsequent generation, suggesting the capacity of cytokinins to affect the phase of the circadian clock. Hormonal regulation of heritably altered diurnal periodicity and environmental responses may provide a developmental and, therefore, evolutionary advantage to plants.

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And finally, this thesis is dedicated to my dad, Wayne Lindsay, who died during my Ph.D. studies. He remains my inspiration, mentor, and guide, who by his passion for science and love of plants, was instrumental in me pursuing this challenge.

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ABA	abscisic acid
ACC	1-AMINOCYCLOPROPANE-1-CARBOXYLATE ACID
ACCox	1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE
ACS	1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE
AG	AGAMOUS
AHK	*Arabidopsis* HISTIDINE KINASE
AHP	HISTIDINE PHOSPHOTRANSFER PROTEIN
AIG1	AVRRPT2-INDUCED GENE 1
amp1	altered meristem program 1
AMT2	AMMONIUM TRANSPORTER 2
ANT	AINTEGUMENTA
AOC	ALLENE OXIDE CYCLASE
AOS	ALLENE OXIDE SYNTHASE
AOX	ALTERNATIVE OXIDASE
AP	APETALA
APRR	*Arabidopsis* PSEUDO-RESPONSE REGULATOR
ARF	AUXIN RESPONSE FACTOR
ARK3	*Arabidopsis* RECEPTOR KINASE 3
ARR	*Arabidopsis* RESPONSE REGULATOR
BA	N^6-benzyladenine
BAP	N^6-benzylaminopurine
BCB	BLUE-COPPER BINDING PROTEIN
CAB	CHLOROPHYLL A/B BINDING PROTEIN
CAF-1	CHROMATIN ASSEMBLY FACTOR-1
CBF	C-REPEAT/DRE BINDING FACTOR
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
CCR2	CINNAMOYL COA REDUCTASE 2
CDF1	CYCLING DOF FACTOR 1
CDPK	CALCIUM- and CALMODULIN- DEPENDENT PROTEIN KINASE

CHS	CHALCONE SYNTHASE
CKX	CYTOKININ OXIDASE
CLF	CURLY LEAF
CLV	CLAVATA
CMT	CHROMONOMETHYLASE
CMT3	CHROMOMETHYLASE 3
CO	CONSTANS
COP	CONSTITUTIVE PHOTOMORPHOGENIC
COR	COLD-RESPONSIVE
CRK11	CYSTEINE-RICH RLK 11
CRY	CRYPTOCHROME
CycD3	CYCLIN D3
CZF1	COLD INDUCED ZINC FINGER PROTEIN 1
DAD1	DEFECTIVE IN ANTHER DEHISCENCE 1
DCL1	CARPEL FACTORY/DICER-LIKE 1
DDE1	DELAYED DEHISCENCE 1
DDM1	DECREASE IN DNA METHYLATION 1
DET	DE-ETIOLATED
DIN	DARK INDUCIBLE
DNMT	DOMAIN REARRANGED METHYLTRANSFERASE
DRE/CRT	dehydration-responsive element/C-repeat
DRM1	DORMANCY-ASSOCIATED PROTEIN 1
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
EIN	ETHYLENE INSENSITIVE
ELF4	EARLY FLOWERING 4
ELIP1	EARLY LIGHT-INDUCIBLE PROTEIN
ERF	ETHYLENE RESPONSE FACTOR
ESR1	ETHYLENE RESPONSE SENSOR 1
ETR1	ETHYLENE RECEPTOR 1
FAS	FASCIATA
FKF1	FLAVIN-BINDING KELCH DOMAIN F BOX PROTEIN 1

FLC	FLOWERING LOCUS C
FRI	FRIGIDA
FT	FLOWERING LOCUS T
GA	GA REQUIRING
GASA	GA-STIMULATED
GAST1	GA-STIMULATED TRANSCRIPT 1
Gcn5	histone acetyltransferase
GH3	GLYCINE HYPOCOTYL 3
GI	GIGANTEA
GO	Gene Ontology
GST	GLUTATHIONE S-TRANSFERASE
HAT	HISTONE ACETYLTRANSFERASE
HDAC	HISTONE DEACETYLTRANSFERASE
HSF4	HEAT SHOCK FACTOR 4
HSP70	HEAT SHOCK PROMOTER 70
HY5	ELONGATED HYPOCOTYL 5
IAA	INDOLEACETIC ACID-INDUCED
ICE1	INDUCER OF CBF EXPRESSION 1
iPMP	isopentenyladenosine-5'-monophosphate
IPT	isopentenyltransferases
ISC1	ISOCHORISMATE SYNTHASE 1
KAPP	KINASE ASSOCIATED PROTEIN PHOSPHATASE
KDE®	Kensington Discovery Edition v1.8.2
KNAT	KNOTTED-LIKE IN *Arabidopsis thaliana*
KYP	KRYPTONITE
LFY	LEAFY
LHY	LATE ELONGATED HYPOCOTYL
LOX	LIPOXYGENASE
LRR	leucine-rich repeat
LTP	LIPID TRANSFER PROTEIN
MBF	MULTIPROTEIN BRIDGING FACTOR

MERI5B	MERISTEM PROTEIN5B
MET1	DNA-METHYLTRANSFERASE 1
MPK	MITOGEN-ACTIVATED PROTEIN KINASE
MRP7	MULTI-DRUG RESISTANCE PROTEIN 7
MSI1	MULTICOPY SUPPRESSOR OF IRA 1
NAP	NAC-LIKE, ACTIVATED BY AP3/PI
NRT2:1	NITRATE TRANSPORTER 2:1
P5CS1	PYROLINE-5-CARBOXYLATE SYNTHETASE
PAD	PHYTOALEXIN DEFICIENT
PAL	PHENYLALANINE AMMONIA-LYASE
PCD	programmed cell death
PCL1	PHYTOCLOCK 1
PDF1.2	PLANT DEFENSIN PROTEIN 1.2
PGIP1	POLYGALACTURONASE INHIBITING PROTEIN 1
PHYA-E	PHYTOCHROMES
PHOT1	PHOTOTROPIN 1
PI	PISITLLATA
PIF	PHYTOCHROME INTERACTING FACTOR
POL	POLTERGEIST
POR	PROTOCHLOROPHYLLIDE REDUCTASE
PR	PATHOGENESIS-RELATED
RAV	RELATED TO ABI3-VP1
RBR1	RETINOBLASTOMA-RELATED 1
RCI2B	RARE-COLD-INDUCIBLE 2B
RHL41	RESPONSIVE TO HIGH LIGHT 41
RT-PCR	reverse transcriptase polymerase chain reaction
SAG	SENESCENCE ASSOCIATED GENE
SAH	S-adenosyl-L-homocysteine
SAM®	Significance Analysis of Microarrays 2.0
SAMeth	S-adenosylmethionine
SAUR	SMALL AUXIN UP RNA

SCR	SCARECROW
SEM	scanning electron microscopy
SEN1	SENESCENCE-ASSOCIATED 1
SEP	SEPALLATA
SHD	SHEPHERD
SHP	SHATTERPROOF
SIB1	SIGMA FACTOR BINDING PROTEIN 1
SID1	SALICYLIC ACID INDUCTION DEFICIENT 1
SNC1	SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1
SPL	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
ST	SULFURTRANSFERASE
STM	SHOOTMERISTEMLESS
SUP	SUPERMAN
SYD	SPLAYED
TAIR	The *Arabidopsis* Information Resource (http://www.arabidopsis.org)
TCH3	TOUCH 3
TFL1	TERMINAL FLOWER 1
TIR	TOLL/INTERLEUKIN-1 RECEPTOR-LIKE
TOC1	TIMING OF CAB 1
TSK	TONSOKU
TT	TRANSPARENT TESTA
ULT	ULTRAPETALA
WOX	WUSCHEL-related homeobox
WSIP	WUS-INTERACTING PROTEIN
WUS	WUSCHEL
YLS9	YELLOW-LEAF-SPECIFIC GENE 9
ZAT12	ZINC FINGER FAMILY 12
ZLL	ZWILLE/PINHEAD
ZTL	ZEITLUPE

1. INTRODUCTION

Cytokinins regulate essential and diverse aspects of plant development and physiology, including DNA synthesis, cell division, seedling de-etiolation, chloroplast biogenesis, apical dominance, branching, flower and fruit development, leaf senescence, and stress tolerance (Letham, 1994; Mok, 1994). These processes are also influenced by other stimuli, so that cytokinin regulation appears to be integrated with factors such as light and other hormones. For example, it has been proposed that cytokinins play a role in photoperiod induction of flowering, a process also regulated by gibberellins (Bernier, 2005). Endogenous cytokinin levels are responsive to photoperiod and temperature (Wang et al., 2004) and show circadian periodicity (Stiebeling and Neuman, 1986). A preliminary investigation of cytokinins as zeitgebers, or clock timekeepers, has been undertaken (Salomé et al., 2006). The vast repertoire of plant defense responses to pathogens involves regulation by most hormone groups (Hare et al., 1997; Schenk et al., 2000) and the role of cytokinins in regulatory crosstalk of these and other processes is considerable and requires further definition. The present study was carried out to analyse the genetics of cytokinin regulation, to confirm diverse hypotheses and observations to date, to identify novel regulatory roles for cytokinins, and to direct future research.

Gene expression analyses have revealed that epigenetic modifications can target specific genes involved in flower development, such as *SUPERMAN* (*SUP*) (Jacobsen and Meyerowitz, 1997), and defense responses, such as those in a pathogen resistance gene cluster on chromosome IV (Stokes and Richards, 2002). A unique aspect of the present study was an exploration of what appears to be epigenetic-based inheritance of cytokinin-induced phenotypes and transcriptomic responses.

A brief synopsis follows of plant development processes in regards to shoot meristems and flowers; plant responses to environment factors, focusing on light, biological clocks, and pathogenesis; signal transduction, with attention to hormone crosstalk and transcription factors; and epigenetic inheritance. All topics are reviewed in the context of cytokinin regulation, where established, and possible mechanisms responsible for epigenetic inheritance, where applicable.

1.1 What is a Cytokinin?

Cytokinins are a hormone group identified in tracheophytes, mosses, and algae, and named for their ability to promote cytokinesis – cell division. Following the isolation of kinetin from autoclaved herring sperm DNA (Miller et al., 1956), naturally occurring plant cytokinins were first extracted from, and named for, *Zea mays* L. (Letham, 1963). Zeatin is the most prevalent of numerous naturally occurring cytokinins, all of which are N^6-substituted adenine derivatives. Cytokinins serve as regulators in balancing aerial and subterranean growth as extrapolated from their capacity, in concert with auxin, to promote shoot and root growth from callus tissue (Skoog and Miller, 1957).

Cytokinin concentrations are highest in locations of synthesis, i.e., actively dividing tissues such as root and shoot apices, buds, cambial tissue, developing endosperm, and young fruit, and low in differentiated organs such as mature leaves (Srivastava, 2002). An especially important location of cytokinin production is root tips, from which they are transported through the xylem to the shoot system, and this synthesis and redistribution are directed by environmental stimuli (Van Staden and Wareing, 1972). Phenotypic responses to increased cytokinin concentrations have been described in relation to shoot proliferation, flowering, light and temperature responses, senescence, and pathogenesis; however, occasional contradictory results have proven difficult to consolidate (Mok et al., 1987; Adamaska and Kloppstech, 1994; Mok, 1994; Deikman, 1997; Werner et al., 2003; Kumar et al., 2004; Bernier, 2005).

Although defined by their biological function rather than structure, cytokinins share an adenine moiety with various side chains. A model for the biosynthesis of some zeatin-type cytokinins features isopentenyltransferases (ipt) catalyzing the transfer of an isopentenyl group from dimethylallyl diphosphate to adenosine-5'-monophosphate, forming isopentenyladenosine-5'-monophosphate (Kakimoto, 2001), the latter serving as a precursor for the many forms of naturally occurring cytokinins (Mok and Mok, 2001). These pathways were largely determined by introduction of ^{14}C-labeled adenine and analysis of products, a method limited by the low abundance of products in a high density radiation background (Chen and Melitz, 1979; Blackwell and Horgan, 1994). IPT enzymes were first identified in pathogens, e.g., gall-forming bacteria such as

Agrobacterium tumefaciens (Smith and Townsend) Conn transfer *ipt* genes to host plants to promote cytokinin production and cytokinesis (Akiyoshi et al., 1984). An enzyme with ipt-function has not been isolated from plants, probably due to instability and susceptibility to phophatase attack. By sequence similarity and recombinant expression, nine genes encoding proteins with IPT-activity have been identified in the *Arabidopsis* genome (Kakimoto, 2001). The lack of cytokinin synthesis mutants has also restricted isolation of associated enzymes and precise steps and products in cytokinin biosynthesis remain at least partially speculative.

Maintenance of active cytokinin concentrations depends on rapid interconversion of the N^9-riboside and ribotide forms, conjugation with O-glycosides (sugars and –OH groups), and cleavage of the N^6 side chain from adenine (Auer et al., 1999). The latter process of cytokinin degradation depends on CYTOKININ OXIDASES (CKXs). Increased cytokinin levels, associated with rapid cell division in specific tissues, such as maturing fruit and galls formed by *Agrobacterium ipt*-transformation, as well as exogenous application, induce cytokinin degradation (Terrine and Laloue, 1980). CKX action is selective as they are unable to cleave side chains lacking double bonds, with glucosyl residues, and aromatic rings. A sub-family of artificial and naturally occurring cytokinins resistant to CKX-activity features ring substitutions at the N^6-position. These stable and physiologically active cytokinins include kinetin, N^6-benzyladenine (BA), and N^6-benzylaminopurine (BAP) (Mok and Mok, 2001), which are often utilized as exogenous agents in physiological and transcriptomic studies.

1.1.1 Cytokinin Perception and Two-component Systems

Until relatively recently, cytokinins were the least understood plant hormones in regards to perception and signal transduction. It has since been shown that cytokinins are perceived by His kinases and increase transcription of response regulators, elements of a phosphorelay system resembling two-component signal transduction systems (Kakimoto, 1996; Brandstatter and Kieber, 1998). These ancient systems for mediating responses to the environment have been described for bacteria, slime mould, fungi, and most recently plants, but not for animals (Stock et al., 2000). In bacteria, two-component His kinase sensors autophosphorylate upon direct perception of environmental signals

such as light, temperature, chemotaxis, oxygen, or osmotic stress (Maeda et al., 1994; Suzuki et al., 2000a). Unlike these bacterial His kinases, which respond exclusively to environmental cues, plant pathways also respond to hormones, in particular cytokinins. Cytokinin involvement in two-component systems has been extensively documented and reviewed (D'Agostino and Kieber, 1999; Suzuki et al., 2001; Hwang et al., 2002; Kieber, 2002; Sheen, 2002; Rashotte et al., 2003).

In *Arabidopsis*, genes sharing sequence similarity with the His kinases of bacteria include cytokinin receptors, the light receptor PHYs, and ethylene receptors. The PHYs and ethylene receptors appear to lack the characteristic transmitter sequence motifs associated with kinase activity so they may not function in phosphorelay (Stock et al., 2000). A family of three cytokinin-inducible *Arabidopsis* HISTIDINE KINASE (AHK) receptors, AHK2, AHK3, and AHK4/CRE1/WOL, have been identified (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). Relative to the two-component systems in bacteria, similar systems in plants appear to be more complex, with intermediate histidyl-aspartyl phosphorelay components participating in His kinase activated phosphorylation cascades (Suzuki et al., 2002). Apparently also functioning downstream in His kinase signalling pathways is a family of *Arabidopsis* RESPONSE REGULATORS (ARRs) (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Hwang et al., 2002). The *Arabidopsis* genome encodes ten A-type ARRs, which fall into five pairs, reflecting gene duplication events (Vision et al., 2001). A-type *ARRs* appear to be cytokinin primary response genes and *ARR4*, *ARR5*, *ARR6*, and *ARR7* are cytokinin-inducible (Rashotte et al., 2003). Overexpression of these A-type *ARRs* has been shown to suppress cytokinin-induction of *ARR6*, indicating negative feedback regulation of their own expression (Hwang and Sheen, 2001; Kim et al., 2006). Although the role of A-type ARRs is not fully defined, they are thought to inhibit transcription, perhaps functioning to limit the transient induction of cytokinin-response genes or fine-tuning signal transduction pathways.

The eleven B-type ARRs encoded in the *Arabidopsis* genome, characterized by a receiver domain and C-terminal extension, are thought to act as transcription factors. The C-terminal end contains a conserved domain, which in ARR1 and ARR2 has been shown to preferentially bind to the sequence 5'-AGATT-3' (Sakai et al., 2000). B-type

ARR1, ARR2, ARR10-14, ARR18-21, and ARR23, featuring MYB-like domains for DNA binding, have been shown to regulate transcription of A-type *ARRs* in a cytokinin-responsive manner (Hwang and Sheen, 2001). Expression of B-type *ARRs* is not affected by cytokinins (Kiba et al., 1999); however, transgenic *Arabidopsis* overexpressing B-type *ARR2* displays characteristics associated with increased cytokinin levels, including cell proliferation, shoot production, and delayed senescence, indicating a role in cytokinin signalling (Hwang and Sheen, 2001).

1.2 Shoot Meristems

Throughout its life cycle, the aerial portion of a plant is generated by shoot meristem activity. The shoot meristem is a highly specialized region maintaining a central core of pluripotent cells, while directing peripheral cells towards differentiation and the generation of organ primordia (Steeves and Sussex, 1989; Clark, 2001; Fletcher, 2002; Bäurle and Laux, 2003). Leaves and floral organs are initiated in a species-specific spatial pattern, giving rise to the phyllotaxy and floral formula of a plant. A vital aspect of shoot meristem development is the conversion from vegetative to reproductive function. In *Arabidopsis*, the meristem becomes broader and domed during the transition to an inflorescent state (Vaughan, 1955). Once conversion to reproductive identity has been achieved, inflorescence meristems produce floral meristems, which in turn initiate organ primordia in an orderly and predictable sequence of centripetal whorls to form a flower (Smyth et al., 1990; Greyson, 1994).

As proposed by Goethe in the eighteenth century, the leaves, flowers, and floral organs generated in angiosperms are homologous to shoots (Arber, 1937; Wilson, 1945; Bowman and Meyerowitz, 1991). This shared ancestry is likely the basis of pivotal regulatory pathways governing meristem-cell maintenance, as directed by *SHOOTMERISTEMLESS* (*STM*) (homeodomain protein), *CLAVATA1* (*CLV1*) (receptor kinase), and *WUSCHEL* (*WUS*) (homeodomain transcription factor), being common to all shoot meristem states (Clark et al., 1993; Endrizzi et al., 1996; Laux et al., 1996; Groß-Hardt and Laux, 2003).

The first gene to be identified with a role in maintaining shoot meristem function was the maize *KNOTTED1* gene (Vollbrecht et al., 1991). In *Arabidopsis*, members of

the *KNOTTED-LIKE IN ARABIDOPSIS THALIANA* (*KNAT*) gene family function in a similar manner by regulating cell differentiation in the meristem (Lenhard et al., 2002). Shoot meristem initiation is regulated in part by *STM*, of the *KNAT* family, which is expressed in the central apical cells of young embryos (Long et al., 1996). Throughout development, STM is thought to competitively oppose CLV1 function by limiting the transition of meristematic cells from pluripotent to differentiated, thereby maintaining the balance of stem-cell population and organ formation (Clark et al., 1996; Endrizzi et al., 1996). It has been suggested that *STM* and *WUS* serve distinct yet complementary functions in the shoot meristem, with *STM* restraining precocious differentiation of meristem cells while *WUS* promotes the maintenance of the central region of pluripotent cells (Carles and Fletcher, 2003).

Hormones serve as important intrinsic regulatory factors, responsive to external conditions, in the coordination of gene expression underlying the differentiation processes in the shoot meristem. Cytokinins and auxin in particular exhibit a regulatory impact on cell proliferation and organ differentiation (Skoog and Miller, 1957); therefore, cytokinin and auxin regulation of meristem activity is implied, but not clearly defined, in regards to the expression of genes governing meristem operation and subsequent floral development (Leyser, 2003). Conversely, a picture is emerging that cytokinin concentrations in the shoot meristem are influenced by genes controlling meristem maintenance and function (Venglat, 1999; Yanai et al., 2005).

The study of cytokinin regulation of shoot meristem genetics has been hampered by the lack of mutants with altered cytokinin biosynthetic pathways. In an attempt to overcome this limitation, plants have been transformed with the *Agrobacterium ipt* gene, which induces cytokinin biosynthesis in pathogen-infected tumours. Tobacco transformed with *ipt* behind the cauliflower mosaic virus 35S promoter produce ectopic vegetative and floral meristems on the surfaces of excised leaves in vitro, suggesting the capacity of cytokinins to initiate meristematic activity (Estruch et al., 1991). Rupp et al. (1999) transformed *Arabidopsis* with *Agrobacterium ipt* controlled by a *Drosophila melanogaster HSP70* promoter. Heat-activated transgenic plants show reduced apical dominance, a robust production of shoot biomass, and an elevation in *KNAT1* and *STM* transcripts, suggesting that cytokinins have a natural role in shoot meristem

establishment and/or maintenance (Rupp et al., 1999). In *35S::STM* transgenic *Arabidopsis*, elevated levels of endogenous *IPT7* transcript and zeatin-type cytokinins suggest that *KNAT* genes, e.g., *STM*, may serve as central regulators of hormone levels in the shoot meristem (Yanai et al., 2005).

A relationship between cytokinins and meristem function has also been suggested by the enlarged meristem and extra floral organ phenotype of *altered meristem program 1* (*amp1*), an *Arabidopsis* mutant of a glutamate carboxypeptidase resulting in elevated cytokinin levels, as well as increased transcript levels of *STM* and *KNAT1* (Chaudhury et al., 1993). Furthermore, lettuce leaves over-expressing *Arabidopsis KNAT1* show a corresponding accumulation of cytokinins (Frugis et al., 2001), and *Arabidopsis* over-expressing an orchid *CKX* show reduced transcript levels of *STM* and *KNAT1* (Yang et al., 2003). Conversely, exogenous BA was not found to affect *STM* transcript levels in *Arabidopsis* (Rashotte et al., 2003). From these studies it is suggested that cytokinins are positioned upstream of *STM* and *KNAT1*, with potential regulatory feedback loops involving hormone metabolism.

A connection between cytokinins and the CLV pathway, controlling floral meristem function in wild type *Arabidopsis*, has also been suggested (Venglat and Sawhney, 1996). Higher than normal dihydrozeatin levels in the inflorescences of *clv1-1* mutants implies that cytokinins may be the responsible regulator for the increased shoot meristem size of the mutant and that conversely, the CLV pathway may serve in limiting cytokinin biosynthesis or transport (Venglat, 1999). Also potentially affecting cytokinin regulation of meristem function is their intracellular distribution. In shoot meristems, immunocytochemical analyses have shown dihydrozeatin and isopentenyladenine to be located mainly in the cytoplasm, whereas zeatin, the most common cytokinin, is localized in the nucleus (Dewitte et al., 1999).

The CLV receptor kinase pathway has been one of the most-studied signal transduction pathways in plants. *CLV1* encodes a transmembrane leucine-rich repeat (LRR) receptor kinase involved in initiating cell differentiation at the periphery of the apical meristem (Clark et al., 1997). Mutations in *CLV1* affect meristem size, leading to inflorescence fasciation and proliferation of floral organs (Leyser and Furner, 1992; Clark et al., 1993). The CLV1 protein is thought to form a heterodimeric receptor

complex with the accessory receptor-like protein CLV2, with CLV3 serving as an activation ligand for the CLV1/CLV2 complex (Jeong et al., 1999). Proper folding and/or complex formation of the CLV proteins results from interactions with the chaperone SHEPHERD (SHD) (Ishiguro et al., 2002). It is thought that the ensuing phosphorylation cascade, activated by a functioning CLV-complex, may be negatively regulated by the protein phosphatase KINASE ASSOCIATED PROTEIN PHOSPHATASE (KAPP) (Trotochaud et al., 1999). Another component of the signal transduction pathway, POLTERGEIST (POL) is proposed to have a role in carrying the signal activation from transmembrane CLV1 into the nucleus, resulting in transcription of the appropriate downstream structural genes (Yu et al., 2003). An important feedback function of the CLV pathway seems to be regulation of the size of the *WUS* expression domain within the stem-cell population (Brand et al., 2000; Schoof et al., 2000). A member of the WUSCHEL-related homeobox (WOX) subfamily of transcription factors, WUS restricts the spatial limits of the pluripotent cells of the shoot meristem (Laux et al., 1996; Haecker et al., 2004; Green et al., 2005; Williams and Fletcher, 2005; Kieffer et al., 2006). Complicating the interpretation of the CLV pathway is an overlap in gene function, for example, the *clv1* mutant phenotype is strongest in the *Arabidopsis thaliana* Landsberg *erecta* background, supporting the assertion that the LRR receptor kinase *ERECTA*, mutated in this ecotype, may be slightly redundant with *CLV1* (Diévart et al., 2003).

The spatial distribution of cells in the shoot meristem is integral to its function. The cells of the central zone of the apex are constrained in a lineage- and position-dependent manner to serve as a reservoir to replenish the peripheral and rib zones, from which primordia and internal tissues are generated, respectively (Steeves and Sussex, 1989). Superimposed over this zonation pattern, shoot meristems of seed plants are generally organized into layers (Foster, 1938). In *Arabidopsis*, three distinct layers are evident with anticlinal divisions of cells within the tunica (L1 and L2) generating epidermal and uppermost subepidermal tissues. The less stratified cells of the corpus (L3) give rise to the bulk of the internal tissues (Traas and Vernoux, 2002). The spatial positioning of undifferentiated stem-cells is determined by several factors, including ZWILLE/PINHEAD (ZLL), a protein conserved in plants and animals, but absent in

single-cell organisms (Moussian et al., 1998). Highlighting the importance of the CLV pathway in the positional determination of cell pluripotency in the shoot meristem, the small, secreted CLV3 is produced in the tunica, while CLV1 is located in membranes of the corpus cells. Typically in *Arabidopsis*, vegetative and inflorescence meristems' expression of *WUS* is restricted to a small group of cells in the corpus, beneath the outer three layers. In floral meristems, *WUS* expression is thought to arise independent of the inflorescence meristem, and is confined to central cells beneath the outermost two meristematic layers (Mayer et al., 1998). In shoot meristems of *clv1* mutants, *WUS* expression expands laterally, spreading deeper into the corpus, and into the second layer of the tunica. Accompanying this spatial expansion, temporal expression of *WUS* persists during carpel formation in *clv1* mutants, by which time it has disappeared in wild type floral meristems (Schoof et al., 2000).

Contradicting the current model of *WUS* expression being regulated by the CLV pathway, Green et al. (2005) reported that *WUS* transcript is patchy or absent in shoot meristems of strong *clv1-4* and *clv3-2* mutants; earlier studies had shown *clv* mutants with an expanded domain of *WUS* expression (Brand et al., 2000; Schoof et al., 2000). Despite a possible diminished presence in the shoot meristems of *clv* mutants, *WUS* expression remains strong in floral meristems, lateral shoot meristems, and developing ovules and anthers, suggesting that *WUS* may be necessary for the initiation of stem-cell identity but not its maintenance (Green et al., 2005).

Regulation of the expression level or domain of *WUS* is associated with the meristematic function of the CLV pathway (Laux et al., 1996), the chromatin remodelling factors FASCIATA (FAS) (Bertrand et al., 2003) and SPLAYED (SYD) (Kwon et al., 2005), the floral organ identity factor AGAMOUS (AG) (Lenhard et al., 2001; Lohmann et al., 2001), and the novel animal-style transcription factor ULTRAPETALA1 (ULT1), which is involved in floral meristem termination (Carles et al., 2004; Carles et al., 2005). Complicating the picture somewhat are data suggesting that WUS also functions as a transcription factor in the induction of at least two of its repressors: *CLV3* (Brand et al., 2000; Schoof et al., 2000) and *AG* (Lohmann et al., 2001).

A structural investigation of WUS has shown that in addition to the homeodomain, the protein includes three conserved C-terminal amino acid sequences: an acidic domain consistent with transcription factor activity, a WUS box (TLPLFPMH) with undetermined function, and an ERF-associated amphiphilic repression domain (ASLELTLN) similar to domains previously shown to function in transcription repression of *SUP* and genes encoding Aux/IAA-responsive proteins (Ohta et al., 2001; Hiratsu et al., 2004; Tiwari et al., 2004). Truncated WUS proteins lacking these C-terminal domains are unable to recruit the transcriptional co-repressors, WUS-INTERACTING PROTEIN 1 (WSIP1) and WSIP2, necessary to inhibit cell differentiation in the shoot meristem (Kieffer et al., 2006). The mechanism of transcription repression has not been determined, although in *Drosophila* histone deacetylation has been suggested (Courey and Jia, 2001). This model is supported by preliminary results in *Arabidopsis* where treatment with the histone deacetylase inhibitor trichostatin A results in phenotypes of meristem termination similar to those seen in *wus-1* (Kieffer et al., 2006).

As meristematic cells exist within a dynamic array of states, ranging from quiescence to differentiated, it is thought that factors involved in chromatin remodelling will be active in apical meristem determination and differentiation. During the course of development, SWI2/SNF2, evolutionarily conserved enzymes at the core of large nucleosome remodelling factors powered by ATP, can affect the accessibility of DNA transcriptional machinery to various cis regulatory elements. The first identified plant SNF2 was *SYD*, which, independent of the CLV pathway, specifically induces transcription of *WUS* (Kwon et al., 2005). Also, *FAS1*, *FAS2*, and *MULTICOPY SUPPRESSOR OF IRA 1* (*MSI1*) encode subunits of the CHROMATIN ASSEMBLY FACTOR-1 (CAF-1) (Leyser and Furner, 1992). *CLV1* was originally designated *FAS3* (Kaya et al., 2001); however, it is now thought that the shared extra floral organ phenotypes of *fas1*, *fas2*, and *clv* mutants are coincidental due to ectopic expression of *WUS* and associated cell proliferation in the shoot meristem of these mutants (Kaya et al., 2001). Spatial expression patterns of *WUS* are thought to be regulated by CAF-1, as an aspect of stabilizing epigenetic inheritance of genes responsible for meristem organization (Kaya et al., 2001). MERISTEM PROTEIN5B (MERI5B), a nuclear

protein, is involved in DNA repair and stabilization, and is proposed to play a role in establishing heritable features of chromatin. Specifically, it has been suggested that *MER15B* may serve in epigenetic inheritance of *WUS* expression patterns (Takeda et al., 2004).

Mutations in *TONSOKU* (*TSK*), expressed in S-phase of the cell cycle and thought to function in DNA repair of gene silencing, also affect *WUS* expression, resulting in patchy *WUS* expression and an enlarged shoot meristem (Suzuki et al., 2004b; 2005). Additionally, the *WUS* expression domain is expanded in mutants of the histone acetyltransferase *GCN5*; however, details of this relationship are not yet clear (Bertrand et al., 2003). The association of *WUS* with chromatin remodelling factors suggests the presence of putative markers for specific epigenetic targeting of histone tails or DNA at the *WUS* locus (Kwon et al., 2005). The implication of *WUS* as a candidate for epigenetic inheritance is of particular significance in this study, as inheritance of an extra floral organ phenotype was observed.

1.3 Flowering Processes

Flowers represent specialized structures of a reproductive phase of shoot meristem activity. The conversion of the meristem from a vegetative to reproductive function and the development of flowers involve direction by environmental factors, sequential expression of integrated classes of genes, and numerous hormones. A defined set number of organs are produced, especially in flowers with a small number of organs such as members of the Brassicaceae family, including *Arabidopsis* (Polowick and Sawhney, 1986; Bernier, 1988; Smyth et al., 1990). In *Arabidopsis*, genes involved in meristem activity and flower development processes have largely been identified by isolation and characterization of mutants (review, Zik and Irish, 2003). Additionally, the role of hormones in flowering has been determined from exogenous applications, endogenous analysis, or transgenic plants with altered hormone metabolism, exaggerating floral phenotypes.

Induction of flowering and flower development are regulated by a large number of genes that may be grouped according to their roles in flower timing, floral meristem identity, and floral organ identity; however, redundancy, overlap, synergy, and interplay

between many of these genes have been noted (Irish, 1999). Genes controlling the initiation and early phases of developmental pathways, such as flower timing, tend to be responsive to dynamic environmental and endogenous cues; in contrast, flower development is a canalized process (Weigel, 1995; Jack, 2004).

1.3.1 Flower Initiation

In order to coordinate reproduction with favourable growth conditions, meristem identity genes integrate endogenous and environmental cues to regulate the timing of flowering. Early in the 20th century it was realized that both the duration and quality of light affect flower timing, and the term photoperiod was introduced (Garner and Allard, 1920). Vernalization, exposure to a long period of cold, was also recognized as inducing flowering in some plants and recently it was determined that this involves altered histone methylation patterns of responsive genes (Medvedev, 1969; Sung et al., 2006). Stresses, such as decreased nutrient or water availability, can also induce reproduction, as seeds are more likely to survive adverse conditions than plants (Levy and Dean, 1998).

Physiological studies have led to several models for the regulation of the transition of the shoot meristem to reproductive function and the subsequent production of flowers (Pharis and King, 1985; Bernier et al., 1990). The florigen theory speculated a substance, which stimulated flowering, was produced in the leaves under facultative short or long days and transported via the phloem to the shoot apex (Lang, 1952); however, despite numerous studies, florigen has yet to be isolated (Evans, 1971). A second hypothesis proposed that nutrient concentrations in the apical meristem induced flowering (Sachs and Hackett, 1983); however, this model was found to be overly simplistic (Bernier, 1988). Currently, an integrated model recognizes regulation by a combination of hormones and assimilates (Bernier, 2005). The complexity inherent in this model can account for the diversity of flowering responses linked to genetic background and/or environmental conditions (Levy and Dean, 1998).

Current studies of environmental and intrinsic promotion of the initiation of flowering generally focus on day length, temperature, and hormones (Reeves and Coupland, 2000; Bernier, 2005). In long day photoperiod-responsive plants, such as *Arabidopsis*, the photoreceptor *CRYPTOCHROME 2* (*CRY2*) has been shown to be a

key element in light-cue flowering; *cry2* mutants flower late under long-day conditions while *CRY2*-overexpressors flower earlier under short-day conditions (Guo et al., 1998; Lin and Shalitin, 2003).

Research into the hormonal regulation of flowering has focussed on gibberellic acid and its activation of *LEAFY* (*LFY*) transcription from promoter sites distinct from those responsible for the photoperiodic responses of *LFY* (Blázquez et al., 1998). Mutants with altered metabolism of, or sensitivity to, abscisic acid, ethylene, brassinosteroids, and salicylic acid have also been shown to affect flowering time, implicating hormone crosstalk in floral initiation pathways (Chory et al., 1991; Martínez-Zapater et al., 1994; Ogawara et al., 2003; Martínez et al., 2004).

Changes in flower timing have long been associated with cytokinin treatments (Nakayama et al., 1962) and physiological studies have indicated a role for cytokinins in the induction of flowering in long day plants (Bernier, 2003). Experiments with exogenous cytokinins to promote fruit production in grapes and bamboo suggest that BA-type cytokinins are sufficient, and possibly required, to induce flowering (Joshi and Nadgauda, 1997). Cytokinins can induce early flowering in wild type *Arabidopsis* (Chory, 1994) and initiate flowering in *Pharbitis nil* Chois. (Ogawa and King, 1980), *Wolffia microscopica* (Griffith) Kurz (Venkataraman et al., 1970), and *Lemna paucicostata* Hegelm. (Gupta and Maheshwari, 1970). Early flowering occurs in the cytokinin-overproducing *Arabidopsis* mutant *amp1* (Chaudhury et al., 1993). In contrast, *Arabidopsis* transformed with *Agrobacterium ipt* behind a *Z. mays HEAT SHOCK PROMOTER 70* (*HSP70*) promoter, showed increased cytokinin levels, with no effect on flowering (Medford et al., 1989).

In the long day plant *S. alba* L., a 16 h photoperiod stimulates a rapid export of sucrose from leaves to shoot and root meristems as well as cytokinins from the roots, via the xylem, to the leaves, and on to the shoot apex. Blocking transpiration, and, thereby, the flow of cytokinins from roots to shoots, inhibits the flowering response and exogenous cytokinins recover the phenotype (Bernier et al., 1993; Bernier, 2003). While decreased cytokinin levels are found in the xylem of *Xanthium* L. plants subjected to a reduced photoperiod (Kinet et al., 1994), in *S. alba*, exogenous cytokinins, in combination with high light intensity and sugars, induce shortened cell cycle stages in

shoot meristem cells, mimicking the effect of exposure to long days (Bernier, 2003). Analysis of cytokinin distribution within a plant, with respect to flower induction, showed short day treatments of *Xanthium* coincided with an accumulation of cytokinin free bases and ribosides in the shoot meristem (Van Staden and Wareing, 1972). Likewise, increased cytokinin levels related to temperature or light induction of flowering were found in *Begonia* L. (Hansen et al., 1988). Furthermore, in *S. alba*, cytokinin content in the shoot meristem was also found to increase, in conjunction with flowering (Jacqmard et al., 2002). Similarly, high endogenous cytokinin levels in the shoot meristem of tobacco are associated with the generation of leaf primordia; however, cytokinin levels decrease to undetectable levels during the transition from vegetative to reproductive function (Dewitte et al., 1999).

Despite physiological evidence implicating cytokinins as regulators of flowering in *Arabidopsis* (Chaudhury et al., 1993), genetic evidence of cytokinins as a flowering stimulant has not been conclusive (Corbesier et al., 2003). For example, it has been suggested that the influence of *KNAT* genes on the ratio of gibberellic acid and cytokinins in the shoot meristem in the context of flowering needs to be determined (Yanai et al., 2005). Inconsistencies in observations of cytokinin action in initiation of flowering may be based on concentration of exogenous agents, spatial and temporal distribution, or plant species. Pending further investigation, inconsistencies in plant responses to cytokinins limits conclusions about their role in flowering (Kinet et al., 1993; Bernier, 2003).

1.3.2 Flower Development

A summary of flower development genetics is necessary to a study of the plant transcriptome. The well-documented ABC model describes the temporal/spatial expression of homeotic transcription factors regulating the development of sepals, petals, stamens, and carpels (Bowman et al., 1991; Coen and Meyerowitz, 1991). In *Arabidopsis*, A-function genes, e.g., *APETALA1* (*AP1*) and *AP2*, specify the development of whorl-one sepals, the C-function gene *AG* specifies whorl-four carpels, and active combinations of B-function genes, i.e. *AP3* and *PISITLLATA* (*PI*), with A-function genes specifying whorl-two petals and B- combined with C-function specifying

whorl-three stamens. Orthologs of these genes were concurrently identified in *Antirrhinum* L., supporting the model as general to angiosperms (Bowman and Meyerowitz, 1991; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994).

The ABC-functioning genes were found to be required, but not sufficient, to direct floral organ identity, leading to the discovery of the D-function genes, including *SHATTERPROOF 1* (*SHP1*) and *SHP2*, encoding factors necessary for ovule development (Favaro et al., 2003). Further complicating the previously straightforward ABC model (now ABCD) are the E-function genes; *SEPALLATA 1* (*SEP1*), *SEP2*, *SEP3*, and *SEP4* serve redundant function and are required in combination with the ABCD genes to specify all floral organs (Angenent et al., 1995; Pelaz et al., 2000; Ditta et al., 2004; Castillejo et al., 2005). With a role in regulating these floral organ identity genes, the floral meristem identity gene *LFY* directs the transition of the shoot meristem from vegetative to reproductive function (Schultz and Haughn, 1991; Weigel et al., 1992).

In addition to floral organ identity function, *AP1* serves a similar function to that of *LFY* in floral meristem identity (Bowman, 1993), and *AP1* is required to suppress the formation of buds in the axils of floral organs (Irish and Sussex, 1990). Some of the regulation of *AP1* expression likely occurs through SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3) a transcription factor that has been shown to bind to the promoter region of *AP1* (Cardon et al., 1997; Pelaz et al., 2001). *CARPEL FACTORY/DICER-LIKE 1* (*DCL1*) encodes a RNA helicase involved in microRNA processing and is thought to play a key role in the posttranscriptional regulation of *LFY*, *AP1*, and *AP2*, by targeting mRNA for degradation (Park et al., 2002; Rhoades et al., 2002).

Other genes influence flowering without being categorized with floral organ identity or floral meristem identity function. For example, the transcription factor AINTEGUMENTA (ANT) is involved in integument development and petal epidermal cell identity. ANT also seems to have a role in floral organ identity by repressing *AG* expression in the second whorl (Elliott et al., 1996; Krizek et al., 2000). Some members of the large NAC family of transcription factors, exclusive to the plant kingdom also function in floral morphogenesis (Souer et al., 1996; Aida et al., 1999; Xie et al., 1999).

This group includes *NAC-LIKE, ACTIVATED BY AP3/PI* (*NAP*), thought to influence the transition from cell division to cell expansion in petals and stamens (Sablowski and Meyerowitz, 1998). Numerous other genes also influence floral development and it is highly probable that more are still to be discovered (review, Zik and Irish, 2003).

Furthering our understanding of their important role in shoot development, cytokinins have been shown to be essential for normal flower development. For example in *Brassica napus* L. (Polowick and Sawhney, 1991) and tomato (Rastogi and Sawhney, 1986), in vitro growth of young flower buds to fertile maturity requires the addition of cytokinins, whereas flowers of a male sterile line of *B. napus* have lower levels of endogenous cytokinins than fertile flowers (Shukla and Sawhney, 1992; Sawhney and Shukla, 1994). While cytokinin deficiencies affect flower fertility, increased cytokinin levels also alter flower development. Exogenous cytokinins produce sepal-petal mosaic organs in tobacco (McHughen, 1982; Estruch et al., 1993) and mosaic organs, extra floral organs, and ectopic bud phenotypes in *Arabidopsis* (Venglat and Sawhney, 1996). It has been speculated that the latter effects were related to cytokinin-altered expression of *CLV* and *AP1*, genes regulating shoot meristem function and floral development (Venglat and Sawhney, 1996). Transgenic tobacco with increased cytokinin levels due to the insertion of the bacteria *ipt* gene showed decreased transcript levels of the floral homeotic gene orthologues *AP3*, *PI*, and *AG* (Estruch et al., 1993).

Chromatin remodelling, i.e. chemical alterations to DNA and/or histones by methylation and/or acetylation affecting the transcriptional accessibility of nucleosomes, has been found to be important in the regulation of flowering processes. In *Arabidopsis*, CURLY LEAF (CLF) is a polycomb-group protein that functions in the maintenance of stable gene expression patterns associated with flowering in shoot meristem cells. Although the mechanism has not been identified, CLF seems to serve in maintaining the repressed state of *AG* in the first two whorls of the flower (Goodrich et al., 1997; Schubert et al., 2005). MET1, a DNA cytosine methyltransferase, also affects specific gene expression patterns in developing flowers (Ronemus et al., 1996). That chromatin remodelling factors influence expression patterns within floral developmental processes, including methylation of DNA bases and histone modification (acetylation, phosphorylation, and methylation), is of particular interest to the present study (section

1.6) as these factors have the capacity to affect expression patterns between generations, a process known as epigenetic inheritance (Tremblay et al., 1995; Li, 2002).

1.4 Cytokinins and Plant Responses to Environmental Conditions

Environmental factors can affect the level, location, and action of plant hormones by altering rates of biosynthesis, inter-conversion, transport, degradation, and cell-sensitivity (McCourt, 2001). Cytokinins are essential plant growth hormones associated with positive plant responses to environmental conditions conducive to growth, and also have a role in stress responses, such as those stimulated by water and nitrogen shortage (Hare et al., 1997). They are especially associated with light stimuli, functioning in the reception and transduction of photo-signals essential to growth (e.g., Fankhauser, 2002). Although not generally regarded as a biotic-stress hormone, investigations into the intricate relationship between cytokinins and pathogen attack has also been initiated (Jameson, 2000; Kumar et al., 2004).

1.4.1 Light

Evolution has fine-tuned light reception with signal transduction systems to control numerous plant processes, including germination, emergence from the soil, leaf movement, neighbour detection, and day length perception. Light cues are also utilized to coordinate members of the same species to flower synchronously and to induce seasonal senescence and leaf abscission. Fifty years ago a correlation was reported between cytokinin action and red light responses; seeds of *Lactuca sativa* L., which normally require light, germinated in the dark when incubated in the presence of cytokinins (Miller, 1956). Exogenous cytokinins or variations in endogenous cytokinin levels have been shown to induce effects that mimic other light responses, such as inhibition of hypocotyl-elongation, conversion of etioplasts to chloroplasts, induction of flowering, and increased cell division in the shoot meristem (Chory et al., 1994; Chin-Atikins et al., 1996; Thomas et al., 1997).

Light is perceived by wavelength-receptors, specifically red/far-red light by PHYTOCHROMES (PHYA-E) and blue/UV-A light by CRY1, CRY2, PHOTOTROPIN 1 (PHOT1), and PHOT2. Photoreceptors influence photoperiodism,

photosystems, phototropism, photorespiration, and flowering (Wang and Deng, 2004). As well as monitoring the direction of light for phototropic response, PHOT1 and PHOT2 are involved in chloroplast movement and stomatal opening, with the signal transduction at least partially involving Ca^{2+} (Huala et al., 1997; Harada et al., 2003). Acting concurrently, both PHYs and CRYs regulate flowering, shoot development, and entrainment of biological clocks. From early research on photoreception, it was thought that the active state of PHY flowed, like sand in an hourglass, to a non-active state, and that this was the basis of light responses (Borthwick and Hendricks, 1960). It was also theorized that light response processes were too complex for such a simple model, and it was proposed that PHY perceptions of light stimuli were linked to altered gene expression (Hamner, 1961). Eventually it was determined that spatial distribution is integral to PHY function. PHYA and PHYB show nucleocytoplasmic partitioning, in a light quality-dependent manner, with cytoplasmic localization in the dark and translocation to the nucleus in the light. The light-activated import of PHYs to the nucleus suggests that photo-response involves altered transcription rates (MacKenzie et al., 1975; Sakamoto and Nagatani, 1996; Lin and Shalitin, 2000; Neff et al., 2000). In contrast, CRYs are constitutive nuclear proteins. Different forms of cytokinins, e.g., zeatin and dihydrozeatin, are also localized in the cytoplasm and nucleus (Dewitte et al., 1999). Therefore, there is the potential for cytokinin-specific, light-related regulation involving coincidental location.

In plants, relatively recent work suggests PHYs may be evolutionary descendants of His kinases, cytokinin-responsive receptor proteins of two-component systems. This suggests a potential pathway for cytokinin regulation of light response systems (Smith, 2000; Hwang and Sheen, 2001). Red light induces the active state of PHY, PHY_{FR}, and dark returns the photoreceptor to the inactive PHY_R state. Red light also elevates levels of ARR4. The direct binding of ARR4 to PHYB is thought to stabilize PHY_{FR}, thereby inhibiting dark reversion (Sweere et al., 2001). Over-expression of *ARR4* in transgenic *Arabidopsis* affected red and far-red light sensitivity, increased root elongation, and delayed the onset of flowering (Sweere et al., 2001). Hormonal regulation of these processes is implied by A-type *ARR* genes, including *ARR4*, being induced by cytokinins (Brandstatter and Kieber, 1998). The interaction between ARR4 and PHYB in effecting

light responses was recently expanded to include a role in the regulation of circadian clocks (Salomé et al., 2006). It has been suggested that further analysis of the kinase functions of PHYA-E may reveal additional instances of convergence between cytokinin-regulated two-component systems and light signal transduction pathways (Hwang and Sheen, 2001; Lohrmann and Harter, 2002).

It has been observed that light and day length serve regulatory roles in the convergence of environmental stimuli and plant physiological responses (Pons et al., 2001; Wang et al., 2004). Light regulates endogenous cytokinin levels by stimulating increased biosynthesis in terminal and lateral shoot meristems of growing tissues in *Solanum andigenum* (Hawkes.) Juz. & Buk. (Wang and Wareing, 1979). Extended light periods also result in increased cytokinin levels in the long-day plants *S. alba* and *Solanum tuberosum* L. (Lejeune et al., 1988; Machácková et al., 1998). Comparisons of *S. alba* to the short-day plant *Xanthium strumarium* L. suggest cytokinin synthesis is photoperiodically controlled, affecting flower timing and shoot growth (Kinet et al., 1993). In conifers, extended photoperiods delayed leaf senescence, a developmental condition associated with increased cytokinin levels (Puttonen and Arnott., 1994; Rosenthal and Camm, 1996). Furthermore, cytokinins have been proposed as mediators of shade acclimation (Pons et al., 2001). Cytokinins also coincidentally increase transcript levels of genes that are induced by light, including elements associated with chlorophyll production and photosynthesis, e.g., chlorophyll a/b binding proteins (Parthier, 1979; Kusnetov et al., 1994; Kimura et al., 2003; Rashotte et al., 2005).

A general aspect of a plant's response to its environmental is the accumulation of a large variety of secondary compounds serving unique roles; for example, flavonoids, a well-described group of secondary metabolites have numerous physiological roles in angiosperms, such as pigmentation to attract pollinators and deterring herbivores and infectious agents (Winkel-Shirley, 2001). By absorbing UV light, flavonoids also serve as sunscreens, protecting plant DNA from radiation-induced mutations (Stapleton and Walbot, 1994). A subclass of flavonoids, anthocyanins are responsible for the red and purple pigmentation of flowers and fruit and are strongly regulated by light, with cytokinins enhancing this regulation (Piazza et al., 2002). Separate treatments with red light or kinetin was found to increase both anthocyanin biosynthesis and accumulation in

Amaranthus caudatus L. and mustard (Koehler, 1972; Ford et al., 1981). The first committed step in flavonoid biosynthesis is catalyzed by *CHALCONE SYNTHASE* (*CHS*), which is induced by cytokinins (Fuglevand et al., 1996; Rashotte et al., 2003). However, despite a long history of research into the association of cytokinin regulation of light responses, many questions remain concerning the intermediate steps between light perception, hormone action, and physiological responses (Thomas et al., 1997).

1.4.2 Biological Clocks

Organisms in all kingdoms anticipate and synchronize physiological processes to the daily rhythm of light and dark cycles via circadian clocks. Numerous examples of circadian rhythms persisting in animals and plants over several generations have been documented, including rats raised in continuous light for 25 generations (Bünning, 1967). Plants have evolved biological clocks regulating oscillating circadian gene expression patterns to coordinate physiological processes with the diurnal periodicity of their primary energy source, the sun. In *Arabidopsis*, a small number of genes maintain clock function and over 500 genes have been identified as oscillating in a diurnal cycle (Harmer et al., 2000). The plant biological clock(s) largely affects diurnal oscillations of genes serving in photosynthesis, but also entrains elements serving in plant responses to environmental fluctuations, such as photoperiod timing of flowering and stress responses (Bernier, 2005; Fowler et al., 2005). Many of the genes associated with flowering processes have oscillating expression patterns (Harmer et al., 2000) and the involvement of the circadian clock in regulation of flowering time has been demonstrated in a variety of species (Vince-Prue, 1983; Fowler et al., 1999; Nelson et al., 2000; Doyle et al., 2002; Schultz and Kay, 2003; Imaizumi et al., 2005).

Circadian rhythms in plants have long been recognized; for example, in 1751 Linneaus designed a garden timepiece based on the opening and closing of flower petals at specific times of the day, regardless of clouds or fluctuating temperatures (Moore-Ede et al., 1982). While circadian regulation maintains predictable oscillations independent of external stimuli, these rhythms can be reset by light and/or temperature signals, known as zeitgebers, to synchronize or entrain internal cycles to changing external conditions (Bünning, 1967). Variations in light and temperature can induce independent

gene responses, suggesting that more than one clock may be involved in circadian entrainment in plants (Michael et al., 2003a).

While PHY signaling affects gene expression related to chloroplast function and anthocyanin production, these effects occur within the context of circadian rhythms (Mohr, 1966; Nagy et al., 1988; Sage, 1992). Furthermore, PHYs themselves exhibit diurnal oscillation of nuclear-cytoplasmic distribution (Kircher et al., 2002). Relatively recently, it has been suggested that both PHY and CRY photoreceptors play integral roles in light-induced function and entrainment of plant biological clocks (Nagy et al., 1993; Somers et al., 1998b; Fankhauser, 2002). Light-altered, reversible states of PHY and CRY molecules seem to be important factors in directing central clock function; however, intermediate regulators are likely involved in the persistence of circadian patterns. Regulatory factors outside of the nucleus are implicated by the continuation of diurnal rhythms in the photosynthetic capacity of enucleated cells of the alga *Acetabularia* L. (Sweeney and Haxo, 1961).

The hormone auxin has been linked to circadian patterns (Vanden Driessche et al., 1996), for example, oscillations in auxin concentration have been observed in leaves of *Hibiscus* L., *Piper* L., and *Coffea arabica* L. (Rama Das et al., 1964; Janardhan et al., 1973), and during flowering of *Chenopodium rubrum* L. (Krekule et al., 1985). Furthermore, circadian fluctuation of auxin levels may be responsible for periodicity in peduncle elongation in *Arabidopsis* (Jouve et al., 1998). In *Sorghum*, endogenous levels of gibberellic acid also show circadian oscillation (Foster and P.W., 1995), as does ethylene (Finlayson et al., 1998). Light cues may be a factor in the latter, as expression and activity of ACC oxidase (ACCox), a primary component of ethylene biosynthesis, is influenced by PHY in *Stellaria* L. (Kathiresan et al., 1996). In *Pennisetum* Nutt., it has been shown that ABA concentrations have an endogenous rhythm linked to water availability, with lowest levels recorded at noon (Henson et al., 1982). It has not been established whether these oscillating hormone levels are involved in clock function or reflect a downstream response to circadian entrainment (McClung et al., 2002). As light stimuli entrain the central clock oscillator, and cytokinins are associated with light responses, it would seem prudent to establish whether cytokinin levels have circadian rhythmicity; few studies have addressed this concern. In carrot and *Populus* L.,

endogenous cytokinin concentrations have been shown to oscillate, with peak levels 7 to 8 hours after sunrise (Hewett and Wareing, 1973; Stiebeling and Neuman, 1986).

Hormone regulation of circadian oscillation has been studied in humans. Melatonin, a modified tryptophan produced by the pineal gland during dark periods, serves as an endogenous zeitgeber with direct action on the circadian pacemaker (Armstrong, 1989; Rajaratnam and Redman, 2002). Melatonin has also been found in plants, algae, and dinoflagellates, where levels oscillate diurnally (Poeggeler et al., 1991; Van Tassel and O'Neill, 2001). Preliminary tests of exogenous melatonin to *C. rubrum* reduced flowering levels (Kolár et al., 2003).

Early research on biological clocks focused on coordinated physiological and morphological processes to optimize energy absorption, stomatal pore opening and leaf movement, thereby maximizing photosynthesis efficiency and minimizing associated water stresses (Bünning, 1967). At that time, it was also realized that for ultimate understanding, circadian systems must be studied at the genetic and molecular level (Hastings and Keynan, 1965). Since then, it has been determined that rhythmic expression of at least 500 genes in the *Arabidopsis* genome is tied to anticipation of environmental conditions, especially light, facilitating circadian exploitation of optimal growth opportunities (Harmer et al., 2000; Davis and Millar, 2001). For example, diurnal entrainment includes a family of CAB genes encoding chlorophyll a/b binding proteins and the small subunit of ribulose-1,5-bisphosphate carboxylase /oxygenase (RubisCO) and RubisCO activase (Kreps and Kay, 1997). The oscillation of photosynthetic genes may have deep evolutionary relevance as circadian rhythms have been documented in cyanobacteria and, therefore, chloroplasts may be maintaining the ancient pacemaker of their bacterial ancestors (Kondo and Ishiura, 1999).

In all organisms, circadian clocks feature a gene circuit with negative feedback involving promotion and repression by transcription regulators (Young and Kay, 2001). Several genes responsible for maintaining clock function have been identified in *Arabidopsis*. *TIMING OF CAB 1* (*TOC1*) (also known as *APPR1*) and *PHYTOCLOCK 1* (*PCL1*) are required for mediating the transcription of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) and together with *Arabidopsis PSEUDO-RESPONSE REGULATOR 3, 5, 7,* and *9* (*APRRs*) are

proposed to quantify and sustain circadian phase and period (Schaffer et al., 1998; Wang and Tobin, 1998; McClung et al., 2002; Michael et al., 2003b; Hazen et al., 2005). Other temporal oscillators enslave additional elements specifying clock function; for example, *CINNAMOYL COA REDUCTASE 2* (*CCR2*) functions downstream of *TOC1* probably to control the phase of yet to be identified oscillating processes (Heintzen et al., 1997). *EARLY FLOWERING 4* (*ELF4*), a gene showing robust oscillating expression, is involved in the accuracy and persistence of circadian rhythms and photoperiod-induced flowering, possibly through inter-relationships with *CCA1* and *CONSTANS* (*CO*), respectively (Doyle et al., 2002). Another member of the *ELF* family, ELF3 can directly bind with PHYB, and is thought to serve as a photoperiod sensor, perhaps inhibiting evening signaling (Covington et al., 2001).

The double mutant *arr3,4* shows a long period and a leading phase shift in the circadian clock, the latter being a characteristic of phyB mutants, suggests A-type ARRs affect circadian clock function by targeting a specific, yet unidentified, clock regulator. That A-type *ARRs*, members of two-component signalling systems that are upregulated by cytokinins, affect circadian clock phase implied a link between cytokinins and circadian clock function. Application of the cytokinin kinetin to *arr3,4* mutants altered the phase of the circadian clock, in a dose dependent manner: low concentrations induced a leading phase and high concentrations a lagging phase. *ARR3* and *ARR4* were interpreted as regulating the period and phase of oscillating patterns, whereas, exogenous cytokinins only alter the phase of the clock (Salomé et al., 2006). Another potential link between the *ARR* family and clock function is *PCL1*, a MYB family transcription factor which shares sequence similarity with the DNA-binding domain of B-type ARRs (Hazen et al., 2005).

The participation of *APRR1,3,5,7,9,* in circadian clock function, has been interpreted as evidence of an evolutionary relationship between two-component signal transduction systems and biological clocks (Sakakibara et al., 2000). APRRs differ from A-type and B-type ARRs in that they lack protein domains and motifs associated with DNA-binding and Asp phosphorylation (Makino et al., 2000; Strayer et al., 2000). Although the molecular function of APRRs has not been determined, their oscillating expression patterns are well established and a model has been developed featuring

APRRs as pacemakers fine tuning the circadian clock period (Makino et al., 2000; Mizuno and Nakamichi, 2005; Nakamichi et al., 2005).

1.4.3 Pathogens

The plant arsenal of genetic and physiological changes to limit and eradicate pathogen attack includes induction of pathogen-specific avirulence (*R-Avr*) genes, accumulation of reactive oxygen intermediates, and production of nitric oxide (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). Another key strategy in pathogen resistance, fortifying plant cell walls, slows pathogen invasion, extraction of nutrients, and infusion of enzymes. Finally, programmed cell death (PCD) plays an important role in the hypersensitive response associated with pathogenesis (Grbic and Bleecker, 1995; Hammond-Kosack and Jones, 1996; Desikan et al., 1998; Thomma et al., 1998).

Hormone regulation of a plant's response to biotic stimuli is complex, featuring pathogen-induced biosynthesis of salicylic acid, ethylene, and jasmonic acid. Little has been reported on the role of cytokinins in pathogen resistance; however, variation in the concentration of cytokinins applied to tobacco tissue cultures has been shown to affect disease resistance (Haberlach et al., 1978). PCD, a complex programme of physiological events involving the regulation of numerous genes and resulting in specific cell death, is a key component of many pathogen defense responses (Smart, 1994) and relatively recently, it has been shown that cytokinins can induce PCD in both plants and animals (Ishii et al., 2002; Mlejnek and Prochazka, 2002). Specifically, BA can directly trigger PCD in *Arabidopsis* suspension cultures, bypassing the intermediate step of ethylene biosynthesis (Carimi et al., 2003; 2004).

PCD can also be invoked by reactive oxygen species and nitric oxide (Delledonne et al., 1998). Nitric oxide is a water and lipid soluble molecule that is involved in several plant processes, including stomatal function, root development, and pathogenesis. Cytokinins have been shown to induce nitric oxide production in cultured cells of *Arabidopsis*, tobacco, and parsley (Tun et al., 2001). Specifically, it has been suggested that cytokinins invoke PCD via a nitric oxide inhibition of mitochondrial respiratory function (Carimi et al., 2005).

The ability to detoxify harmful compounds is also an essential aspect of plant defense responses. A strategy for detoxification of electrophilic compounds features three groups of enzymes. The pathway begins with transformation enzymes, such as cytochrome P450s introducing functional groups onto the substrates, then conjugation enzymes, including GLUTATHIONE *S*-TRANSFERASES (GSTs), utilize the functional group for further conjugation, and finally compartmentation enzymes, such as membrane pumps, sequester the conjugates to vacuoles or the apoplast (Sandermann, 1992; Marrs, 1996).

With 256 genes encoding putative cytochrome P450s, this is one of the largest families in the *Arabidopsis* genome. These monooxygensase enzymes catalyze diverse reactions based on the activation or reduction of molecular oxygen (Mansuy, 1998). Biological functions of cytochrome P450s in plants are predominately associated with pathogen attack, featuring metabolism of xenobiotic compounds. They can also serve in the biosynthesis of flavonoids, antioxidants, gibberellins, auxin, brassinosteroids and jasmonates (Werck-Reichhart et al., 2002). *GSTs* are a family of multifunctional, dimeric enzymes thought to have evolved to protect cells from reactive oxygen species resulting from various biotic stress responses (Hayes and McLellan, 1999). GSTs recognize and metabolize compounds of both exogenous 'xenobiotic' and endogenous origin. Individual *GSTs* are differentially induced by herbicides, biotic elicitors, ozone, hydrogen peroxide, heavy metals, heat shock, dehydration, wounding, and senescence (Marrs, 1996). In the last stage of dexoification, protein pumps embedded in the tonoplast sequester toxic compounds, e.g., herbicides and secondary metabolites, including anthocyanins, into the vacuole (Martinoia et al., 1993; Marrs and Walbot, 1997). A subfamily of *GST* genes have been shown to be auxin-inducible and some auxin-binding proteins are GSTs (Zettl et al., 1994b). That GSTs serve as auxin- and cytokinin-binding proteins has been interpreted as implicating hormone regulation of their activity (Zettl et al., 1994a; Gonneau et al., 1998).

1.4.4 Low Temperatures

Temperate plants, sensitive to low temperatures, are susceptible to wilting, loss of chlorophyll, and restricted growth when the temperature dips below chilling (13°C) or

cold (4°C) temperatures (Wang, 1990). In plants that have evolved temperate climate adaptations, an increase in freezing tolerance, known as cold acclimation, mediates the associated stresses by the accumulation of proline and/or other cryoprotectants, as well as active oxygen species detoxification elements, altering membrane composition, and changes in carbon metabolism (Thomashow, 1999; Iba, 2002). The first step in cold acclimation is recognition of the low temperature, which, in *Arabidopsis*, may involve a two-component signal transduction system such as the temperature sensitive *AHK1* (Maeda et al., 1994; Urao et al., 1999). This likely evolved in temperate regions, where light and temperature are seasonally linked.

Studies of the integral role of transcriptional regulation in low temperature acclimation (Guy et al., 1985) and drought response have identified a promoter sequence DRE /CRT: CCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994) important for the rapid induction of downstream components of cold acclimation induced by the AP2-domain transcription factors C-REPEAT/DRE BINDING FACTOR 1-3 (CBF1-3) (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). Many of the CBF regulon of approximately 45 genes feature the DRE/CRT element in their 1 kb upstream region (Fowler and Thomashow, 2002). Some of these genes are expressed transiently, while others persist at high levels for a week or longer after exposure to low temperatures (Fowler and Thomashow, 2002). Included in the CBF1-3 regulon are the *COLD-RESPONSIVE* (*COR*) genes, one function of which is to decrease the tendency for membranes to be damaged by configuration changes incurred by desiccation stresses associated with cold, drought, and salt. Specifically, *COR15a* encodes a polypeptide targeted to stromatal compartments where it serves to minimize formation of deleterious hexagonal II phase lipids upon cold-induced dehydration (Steponkus et al., 1998).

Exogenous ABA increases freezing tolerance in potato and furthermore, cool temperatures were found to increase ABA levels (Chen et al., 1983). Transcriptome analyses of cold-treated plants have shown a downregulation of auxin-responsive and brassinosteroid-biosynthesis genes, and upregulation of genes associated with jasmonic acid and ethylene (Hannah et al., 2005). To date there has been no evidence to link cytokinins and cold responses, however, the response regulator *ARR4* is upregulated by cytokinins and also transiently induced by cold temperatures (Urao et al., 1998).

1.5 Signal Transduction

Plant processes utilize signal transduction pathways to implement reversible physiological changes specific to dynamic extrinsic conditions. These pathways, commonly composed of kinase receptors, phosphorylation relays, and transcription factors, generally begin with hormone induction of a conformational change in a receptor protein and end with altered gene expression. (McCourt, 1999).

1.5.1 Hormone Crosstalk

Plant hormones function alone, and in combination, to regulate sensory systems and orchestrate appropriate adaptive responses to biotic and abiotic stimuli. Based on his work on the generation of roots and buds from plant callus, Skoog (1994) was one of the first researchers to determine that plant hormones work interactively throughout development. Plants have evolved complex hormonal regulatory interconnections facilitating developmental efficiency and plasticity in growth, reproduction, and response to the environment. An ongoing challenge for plant biologists is deciphering the multi-functional aspect of specific hormones in regulating seemingly unrelated processes and, at the same time, determining how different hormones trigger identical responses. The solution seems to lie in two aspects of hormone regulation. First, hormones have demonstrated the ability to induce changes in synthesis or degradation of other hormones and in the sensitivity of cells to hormone signals. Second, signal transduction pathways include components responsive to multiple hormones. For example, environmental stresses commonly invoke shared early responses of transcript level changes, which often become stimulus-specific within 24 hours (Kreps et al., 2002; Zhu and Provart, 2003). This complex physiological control network, referred to as crosstalk, incorporates regulation of coincident and specific signal transduction pathways (McCourt, 2001). Although crosstalk largely occurs at the level of specific protein-protein interactions, much can be inferred from the study of gene expression patterns.

1.5.1.1 Cytokinins and Auxin

One of the best-documented hormone-hormone interactions in plants is the

relationship between cytokinins and auxin. Cytokinins were originally identified as growth factors by their ability to act synergistically with auxin to promote cell division and antagonistically with auxin to promote root and shoot initiation in callus in vitro (Miller et al., 1955). In vivo, concentrations and ratios of auxin and cytokinins define plant growth and architecture through controlling degrees of shoot apical dominance, initiation of lateral roots, regulation of senescence, and activation of phloem and xylem differentiation (Aloni, 1995; Coenen and Lomax, 1997; Swarup et al., 2002; Ellis et al., 2005). To maintain a dynamic ratio of cytokinins to auxin, relatively large quantities of cytokinin glycosides and various conjugates of indole acetic acid bound to polysaccharides serve as rapidly convertible hormone precursors in a sophisticated mechanism for controlling effective concentrations (Cohen and Bandurski, 1982; Shaw, 1994). Exogenous auxin can directly affect cytokinin metabolism by decreasing expression of *CKXs* (Rashotte et al., 2003; 2005). Exogenous cytokinins can increase transcript abundance of genes induced by auxin, including *INDOLEACETIC ACID-INDUCED* (*IAAs*), *SMALL AUXIN UP RNA* (*SAURs*) and *GLYCINE HYPOCOTYL 3* (*GH3s*), indicating coordinated regulation between these two growth hormones (Rashotte et al., 2005). Mutants with altered auxin and cytokinin signalling have helped to identify convergent regulation points in hormone crosstalk (Frank et al., 2000; Harrar et al., 2003). While it has been established that cytokinins and auxin affect each other's metabolism and/or actions, much remains to be determined about how this translates into coordinating developmental processes at the genetic level (Rashotte et al., 2005).

1.5.1.2 Cytokinins and Ethylene

The gaseous hydrocarbon plant hormone ethylene functions in a myriad of roles in plant processes, pertaining to seedling emergence, flowering, organ abscission, fruit ripening, senescence, and resistance to abiotic and biotic stresses (Mattoo and Suttle, 1991). The ethylene biosynthetic pathway includes the conversion of S-adenosyl-methionine to 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) by ACC SYNTHASE (ACS) and ends with the production of ethylene from ACC by ACCox (Yang and Hoffman, 1984; Kende, 1993). Expression/function of *ACS* genes, key components in ethylene production, have been shown to be increased by developmental,

environmental, and hormonal signals, including auxin and cytokinins (Yang and Hoffman, 1984; Vogel et al., 1998; Bleecker and Kende, 2000). Exogenous auxin can elevate transcript levels of six of the eight *ACS* genes in *Arabidopsis*, while exogenous cytokinins are found to negatively regulate ubiquitin/26S proteasome degradation of ACS5 (Tatsuki and Mori, 2001; Chae et al., 2003; Yamagami et al., 2003). It has also been shown that cytokinins act synergistically with brassinosteroids to modulate ethylene biosynthesis (Woeste et al., 1999).

Five proteins with His kinase domains, *ETHYLENE RECEPTOR 1* (*ETR1*), *ETR2*, *ETHYLENE RESPONSE SENSOR 1* (*ESR1*), *ESR2*, and *ETHYLENE INSENSITIVE 4* (*EIN4*), are thought to function as ethylene receptors in *Arabidopsis* (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). A similar ethylene-binding receptor protein found in cyanobacteria suggests an ancient evolutionary origin of these proteins (Rodriguez et al., 1999). In *Arabidopsis*, the potential kinase activity of these ethylene receptors may involve two-component systems suggesting that these pathways may serve as a mechanism for hormone crosstalk between ethylene and cytokinins (Inoue et al., 2001).

Research into hormone regulation of stress responses has traditionally focused on ABA, ethylene, and salicylic acid (Bonetta and McCourt, 1998; Leung and Giraudat, 1998; Wildermuth et al., 2001; Schaller and Kieber, 2002). More recently a role for cytokinins in coordinating stress responses has been recognized, although it is realized that crosstalk is likely integral to this function (Urao et al., 1998). Cytokinins are one of many factors that induce the biosynthesis of the gaseous hormone ethylene (Abeles et al., 1992), thought to be a regulator of early development in photosynthetic organisms (Bleecker, 1999) and important to stress responses (Hoffman et al., 1999).

1.5.2 Kinase Signalling

Over 1000 receptor-like kinases have been identified in the *Arabidopsis* genome, based on protein structure of extracellular, transmembrane, and kinase domains. The exponentially higher number of kinases in plants than in animals and fungi may reflect the integration of environmental factors in plant development (Wang et al., 2003). A general link between cytokinins and kinase signalling is suggested from transgenic

Arabidopsis with depressed cytokinin levels which show early termination of leaf cell division and/or differentiation, resembling the characteristics of plants over-expressing *KRP2*, a kinase inhibitor (Werner et al., 2003). His-kinases of two-component systems are especially important elements in cytokinin signalling (section 1.1.2).

Calcium (Ca^{2+}) serves as a ubiquitous secondary messenger in plant signalling of abiotic and biotic stresses, as well as hormonal and developmental processes (Cheng et al., 2002; Chinnusamy et al., 2004). CALCIUM DEPENDENT PROTEIN KINASES and CALMODULIN-DOMAIN PROTEIN KINASES (CDPKs), of vascular and nonvascular plants, green algae, and some protozoa, are defined by a signature, but highly variable, N-terminal serine/threonine kinase domain fused to a Ca^{2+} binding site that provides response sensitivity to concentration, duration, and localization of cytosolic Ca^{2+} levels in developmental and stress pathways (Cheng et al., 2002; Rutschmann et al., 2002; Hrabak et al., 2003; Ludwig et al., 2004). *Arabidopsis* has a large subfamily of 34 CDPKs involved in response to a variety of stimuli, including light intensity, cold-stress, freezing-tolerance, salinity, pathogen attack, wounding, and drought (Urao et al., 1994; Chico et al., 2002). As Ca^{2+} is ubiquitous in stress signalling, it is thought that CDPKs may serve as important nodes in crosstalk between the various response pathways (Ludwig et al., 2004). Changes in cytosolic Ca^{2+} concentrations often occur during hormone signalling. Also, hormonal regulation of some CDPKs occurs at the transcription level, specifically, cytokinins, gibberellic acid, and ABA have been shown to induce *NtCDPK1* in tobacco leaves (Mee Yoon et al., 1999).

Relatively little is known about the function of MITOGEN-ACTIVATED PROTEIN KINASES (MPKs) in plants; however, it has been suggested that they play roles in hormone activation of responses to biotic and abiotic stresses and developmental regulation (Ligterink and Hirt, 2000). That components of MPK cascades can respond to more than one type of stress suggests that these members may act as convergence points in stress signalling; for example, expression of *MPK4* is regulated by cold, low humidity, osmotic stress, touch, and wounding (Ichimura et al., 2000). Currently, it is not known whether MPK- and CDPK- mediated responses interact or diverge (Ludwig et al., 2004); however, *MPKs* have been linked to the abiotic stimuli cold, heat, and drought, and as with CDPKs, MPK cascades may be regulated by auxin, gibberellic

acid, ethylene, and ABA (Mizoguchi et al., 1994; Johri and Mitra, 2001). In tobacco, silencing of the MPK *WIPK* was shown to increase salicylic acid production, whereas the induction of *WIPK* increased jasmonic acid levels (Seo et al., 1999). A relationship between cytokinins and MPKs has yet to be determined.

1.5.3 Transcription Factors and Promoter Motifs

Signal transduction pathways generally conclude in altered gene expression patterns. Approximately 6% of the *Arabidopsis* genome is dedicated to transcription regulators; these 1500+ genes have been grouped into 56 families based on their DNA binding domains (http://datf.cbi.pku.edu.cn/) (Riechmann et al., 2000). Promoter motifs are the transcription factor docking sites and are arguably as important for gene function as the gene's encoded amino acid coding sequences (Wray et al., 2003). Genes with coincidental expression profiles often share consensus promoter motifs and respond to common transcription factors. Specific transcription factors and/or promoter motifs are associated with numerous plant processes, including hormone regulation, abiotic stimuli, and entrained circadian oscillation (Yamaguchi-Shinozaki and Shinozaki, 1994; Janaki and Joshi, 2004). Gene duplication, together with mutations in gene regulatory regions affecting transcription factor binding sites and altering temporal or quantitative expression, are thought to have served as a mechanism for relatively rapid evolution in plants, accommodating the sessile lifestyle (Wray et al., 2003; Gu et al., 2004).

1.6 Epigenetic Inheritance

It has long been recognized that external factors have an integral influence on plasticity of plant developmental processes and plants generate a range of phenotypes in various environmental conditions (Dobzhansky, 1951). Waddington (1953) coined the term 'epigenetics' for changes in gene expression responsible for the causal relationship between differentiation and the emergence of new properties via cell, tissue, or organ specialization during individual development, i.e., the processes connecting the genotype with the phenotype within environmental conditions. A key aspect of early epigenetic studies was to understand how the dynamic genome could be affected by intracellular substrates and extracellular factors during the ontogeny of an individual (Løvtrup, 1974).

Physiological conditions affecting the development of an individual do not, for the most part, make a heritable impact on the genome. Some of the first mechanisms recognized as causing induced inheritance were activation of transposable elements, somaclonal mutation in tissue culture, and DNA sequence amplification (McClintock, 1984; Roth et al., 1989; Stark et al., 1989). Although not fully understood, epigenetic inheritance has been documented in various organisms for decades. For example, in 1961, Waddington found heat-shocked *Drosophila* developed crossveinless phenocopies that bred true after the 12^{th} generation, in the absence of heat shock.

In the 1990's, investigations into the mechanisms of epigenetic inheritance focussed on the relationship between chromatin remodelling and altered gene expression. Chromatin is a complex structure that stores and directs gene activity via shifts in cellular biochemical equilibria (Kornberg, 1974). These shifts can give rise to structural changes in the chromatin ranging from highly compact and repressive to open and expressive. Hypermethylation of a 5' regulatory DNA promoter region of a gene is generally associated with gene silencing, whereas increased methylation in the open reading frame is not likely to affect transcription of a gene (Spena et al., 1983; Bianchi and Viotti, 1988; Jacobsen and Meyerowitz, 1997). The addition or removal of a methyl group to DNA cytosine bases or histone H3 lysine 9 and acetylation of lysine residues of core histones are important mechanisms impacting chromatin state and as a consequence, transcription regulation (Li et al., 1993a; Finnegan and Kovac, 2000; Jenuwein and Allis, 2001; Martienssen and Colot, 2001; Paszowski and Whitham, 2001; Richards and Elgin, 2002). In general, phosphorylation, methylation, ubiquitination, and acetylation of nucleosome components are responsible for biochemical alteration of chromatin, regulating accessibility of the DNA to binding proteins such as transcription factors (Finnegan and Kovac, 2000; Tian and Chen, 2001).

Regulation of gene activity by methylation is predominantly a result of the methylation of specific cytosines bases, which is sequence and stimulus specific; therefore, mechanisms must exist to target methylation and demethylation of the specific cytosine sites (Finnegan and Kovac, 2000). Cytosine methylation seems to be integral to the regulation of gene expression in organisms such as *Homo sapiens* L.*, Mus musculus* L. and *Arabidopsis*; however, it may not be universal, as it has not been found to be

important in *Drosophila melanogaster* Meigen*, Saccharomyces cerevisiae* Meyen ex. EC Hansen, and *Caenorhabditis elegans* (Urieli-Shoval et al., 1982; Proffitt et al., 1984; Simpson et al., 1986). To date, study of chromatin remodelling in relation to gene expression has been largely restricted to mammalian and plant model organisms. Interest in chromatin remodelling has benefited from a connection to stem-cell function, cancer, and aging. For example, evidence indicates a link between mammalian cancers and DNA demethylation with local hypermethylation in tumor-suppressor genes (review, Schulz, 1998). Also, the study of chromatin remodelling in plants was inadvertently bolstered by the discovery that transgenic plants with multiple transgene insertions often experienced gene silencing by de novo methylation (Matzke et al., 2000).

In contrast to animals, the late divergence of the germ line in plants, from persistent meristematic centres, facilitates epigenetic inheritance of chromatin structures modified in response to environmental conditions (Holliday, 1990). Inherited epigenetic states in plants have been documented including transposable elements in *Z. mays* and other plants, methylation patterns of *PHOSPHORIBOSYLANTHRANILATE ISOMERASE* (*PAI*), *SUP*, *AG,* and *FWA* in *Arabidopsis*, and multiple copies of transgenes in tobacco (McClintock, 1967; Brutnell and Dellaporta, 1994; Schlappi et al., 1994; Bender and Fink, 1995; Park et al., 1996; Jacobsen and Meyerowitz, 1997; Jacobsen et al., 2000; Soppe et al., 2000; Jackson et al., 2002a).

Important genes/proteins in the establishment and maintenance of methylation and acetylation patterns in *Arabidopsis* includes *DECREASE IN DNA METHYLATION 1* (*DDM1*), *MET1*, *DOMAIN REARRANGED METHYLTRANSFERASE 1-3* (*DNMT*), *CHROMONOMETHYLASE 3* (*CMT*), *KRYPTONITE* (*KYP*), *CAF-1*, and *GNC5* (Vongs et al., 1993; Cao and Jacobsen, 2002; Jackson et al., 2002a). *METI* is normally expressed in both vegetative and floral tissues, with highest expression levels in apical meristems (Ronemus et al., 1996). Transgenically silenced *MET1* lines show ectopic expression of *AG* and *AP3*, suggesting that spatial/temporal expression of some floral genes is regulated either directly or indirectly by methylation (Finnegan et al., 1996).

Mutations in DNA-methyltransferases in mice can cause abnormal development and embryonic abortion (Li et al., 1992). In contrast, *Arabidopsis ddm1* mutants show a 70% reduction in methylation without exhibiting significant phenotypic abnormalities,

making this hypomethylation mutant particularly valuable in the study of methylation patterns (Vongs et al., 1993). *DDM1* preferentially methylates DNA in heterochromatin, although hypomethylation in *ddm1* was found to be focussed on individual genes over multiple generations (Jeddeloh et al., 1999). Self-fertilization of *ddm1* increased the level of global hypomethylation over several generations, indicating that de novo altered methylation patterns can be established and inherited in *Arabidopsis* (Stokes and Richards, 2002). Within the hypomethylated background of *ddm1* mutants, *SUP* and *AG* are targeted for hypermethylation. Pyrimidine-rich DNA sequences found in the promoter and intron regions of both genes are speculated thought to be the targets for methylation, perhaps because secondary structures, such as hairpin loops, may be involved. In contrast, *LFY*, a flowering gene with a similar level of CT-couplets, but not thought to form secondary structures, was not found to have an increased level of methylation in *ddm1* (Jacobsen et al., 2000).

Chromatin remodelling, with the potential for epigenetic inheritance, has been documented in relation to responses to environmental conditions. The bottom arm of chromosome IV of *Arabidopsis* features a cluster of pathogen resistance genes prone to epigenetic modifications (Stokes and Richards, 2002). Methyltransferase function is cold-sensitive; therefore, low temperature may induce demethylation (Burn et al., 1993). A link has also been noted between light and the induction of chromatin remodelling. Methylation-regulated expression levels of *CHS*, affecting anthocyanin biosynthesis, alter petal colour in petunia (Jorgensen, 1995). Possibly by a similar mechanism, light-induced anthocyanin production in *Z. mays* is linked to variation in caryopsis colour (Cocciolone and Cone, 1993).

Hormone regulation of chromatin states has been investigated. In shoot meristems of peach trees, 5-methylcytidine-immunocytolabelling has determined that the central zone of mature meristems is in a hypermethylated state relative to the juvenile stage. These mature meristematic cells coincidentally showed increased zeatin levels (Bitonti et al., 2002). Furthermore, exogenous BAP induced a 30-80% increase in global DNA methylation in wheat seedlings (Vlasova et al., 1994). While the mechanism responsible was not established, cytokinins have been shown to induce heritable aberrant floral phenotypes in *B. rapa* L. (Blahut-Beatty, 1999).

1.7 Methods to Study Cytokinins

Cytokinin regulation of plant growth and development has been studied in numerous ways: exogenous treatment of various species with correlation of resulting phenotypes to known mutants (Jeffcoat, 1977; Venglat and Sawhney, 1996; Blahut-Beatty et al., 1998); analyses of cytokinin-overproducing or -responsive mutants (Chaudhury et al., 1993; Deikman, 1997); transgenic plants with increased (Estruch et al., 1993; Rupp et al., 1999; Huang et al., 2003) or decreased cytokinin levels (Werner et al., 2003). Currently, one of the most accessible and comprehensive methods to study hormone regulation is at the transcriptome level (Provart and McCourt, 2004).

Theoretically, a study of the role of cytokinins in plant development would be best accomplished by the use of mutants or transgenic plants with altered levels of cytokinin biosynthesis. Unfortunately, mutants with altered cytokinin biosynthesis are limited. The *Arabidopsis* mutant *amp1* has high cytokinin levels; however, as *AMP1* encodes a glutamate carboxypeptidase (Helliwell et al., 2001), these mutants are not suited to a study solely of cytokinin metabolism. While nine *IPT* genes have been proposed as functioning in cytokinin biosynthesis, their actual roles have yet to be determined, and to date no cytokinin-deficient mutants have been identified, possibly because of functional redundancy between biosynthetic pathways. Due to difficulties in altering the metabolism of endogenous cytokinins in a controlled manner, exogenous treatment remains an important method of studying cytokinin effects. Zeatin is the most common cytokinin produced by plants; however, BAP is often chosen for exogenous treatments as it represents a naturally occurring cytokinin (Nandi et al., 1989) inciting strong biological activity and developmental responses.

The model plants that have predominantly been used for physiological experiments, such as *Sinapsis*, *Lycopersicon* L. (tomato), *Nicotiana* L. (tobacco), *Lactuca* (lettuce), *Amaranthus*, and *Poa* L., have limited available genetic information. Whereas *Arabidopsis thaliana*, a small flowering plant discovered by Johannes Thal in the Harz Mountains of Germany in the 16th century has become a widely utilized model organism for the study of genetic and molecular aspects of plant biology. *Arabidopsis* offers an important advantage to research in plant genetics: the availability of

commercial microarrays representing over 22,000 genes (Affymetrix 22k GeneChips®). Other beneficial characters include a small genome size (approximately 125 Mb) that was sequenced in 2000, extensive chromosome mapping, a 6-week life cycle, and small physical stature for easy cultivation (TAIR 2005). Although *Arabidopsis* is not of agronomic significance, as a member of the Brassicaceae it is closely related to crop plants such as canola, mustard, and broccoli. Several *Arabidopsis* ecotypes are employed in research laboratories; *Arabidopsis thaliana* Landsberg *erecta* features robust erect morphology, which facilitates examination of floral phenotypes. Also, numerous mutant lines are available in this background. Based on comparisons with phenotypes of mutants and transgenic overexpression lines in *Arabidopsis*, cytokinin-altered phenotypes can be aligned with changes in transcript levels.

In the decade since microarray technology was first introduced to plant biological research (Schena et al., 1995) there has been an exponential increase in its use in gene expression studies (Bevan and Walsh, 2005). Because hormones affect a complex regulatory system including hundreds to thousands of genes, microarrays are a powerful tool for discerning changes in transcript levels associated with the coordination and integration of environmental stimuli and developmental processes. Raw lists of genes with statistically significant changes in transcript levels, featured in preliminary microarray studies, can be of limited use in understanding the intricate role of gene expression pathways in plant biology. Recently, microarray studies have evolved to synthesize transcript data of relevant individual genes of interest, into extrapolations of the pathways, families, and chromosome groupings involved (Blasing et al., 2005; Schmid et al., 2005; Vanderauwera et al., 2005), providing preliminary data on transcriptomic responses to hormones, including cytokinins (Che et al., 2002; Howell et al., 2003; Rashotte et al., 2003). However, much remains to be determined, especially in correlating cytokinin regulation of gene expression with physiological processes, plant development, and responses to the environment.

1.8 Objectives

The objective of this dissertation was to investigate the *Arabidopsis* transcriptomic responses to exogenous cytokinin. Focus was on cytokinin-induced expression of genes regulating shoot meristem function, flowering processes, responses to environmental factors, and signal transduction. Also, novel to this study was an exploration of the inheritance of cytokinin-induced floral phenotypes and epigenetic inheritance of transcript levels altered by the cytokinin treatment.

2. MATERIALS AND METHODS

2.1 Plant Growth

Seeds of *Arabidopsis thaliana* Landsberg *erecta*, supplied by Dr. G.W. Haughn (University of British Columbia) and *clv1-1* and *amp1* mutants by *Arabidopsis* Biological Resource Center (ABRC), Ohio State University, Columbus, OH, were planted in plastic pots filled with RediEarth, watered from below, fertilized once with 2.5 g 20-20-20, and grown at 16 h light by flourescent tubes and incandescent bulbs at an intensity of 120-150 μE m^{-2} s^{-1} and temperature at 23ºC ±1º/8 h dark 19ºC ±1º in Conviron growth chambers. BAP (Sigma-Aldrich Co, St Louis MO) in 50 μl 1 M NaOH was warmed until dissolved, then diluted in 50 ml sterile distilled water to 10^{-3} M, a concentration known to induce aberrant floral phenotypes in *Arabidopsis* (Venglat and Sawhney, 1996). Three μl of 10^{-3} M BAP in 0.05% $Tween_{20}$ (Sigma-Aldrich Co, St Louis MO) was applied with a Hamilton syringe to the surface of the shoot apex of plants when 4-5 rosette leaves were ≥ 1 mm, shortly after the time of transition from a vegetative to inflorescence meristem. The phenotypes of the proximal 15 flower positions in the racemes of control and hormone-treated plants were recorded and photographed.

Second generations (T_1) of plants were grown from seed harvested from the first five flower positions of BAP-treated plants showing aberrant floral phenotypes. BAP was not applied to T_1 plants. Subsequent non-treated generations (T_2, T_3, T_4, T_5) were also grown. In a separate experiment, a series of three consecutive generations were treated with BAP, followed by a non-treated generation. *Arabidopsis* populations were also grown in a low temperature regime of 18/13°C 16/8 h light/dark (control temperature regime 23/18°C day/night). Plants were treated with BAP, as above. Second generations of control and BAP-treated plants (T_1) were grown in the same low temperature regime. Floral phenotypes were recorded.

Exogenous BAP was applied after the transition from a vegetative to an inflorescence meristem; therefore the effects of cytokinin treatment on timing of flowering were measured in T_1 offspring of BAP-treated plants. Flowering time was

measured as the number of rosette leaves at the time that the bolt was 1-2 cm high. Chi square test of homogeneity was performed on the data.

2.2 Scanning Electron Microscopy

Pre-bolting shoot meristems, closed buds, and flowers at anthesis were fixed for scanning electron microscopy (SEM) by vacuum-infiltration with 3% glutaraldehyde in 0.05% cacodylate phosphate buffer (pH 7.0), followed by rinsing in distilled water, and dehydration in a graded acetone series on ice, for 15 min per 10% increment to 100%. Samples were critical-point dried in liquid carbon-dioxide, mounted on aluminium stubs, coated with gold in an Edwards S150B sputter-coater, and observed with a Phillips 505 scanning electron microscope (modified from Venglat, 1999). Contrast and brightness of all photos were adjusted using Adobe Photoshop.

2.3 Transcriptome Studies

2.3.1 Microarrays

Leaves were removed from plants 48 h after BAP treatment and the axes, including shoot and root apical meristems, were flash-frozen in liquid nitrogen. For each array, 150 treated plants and an equal number of control plants were collected from each population. Non-harvested plants from each population were allowed to mature and phenotypes recorded. Plants were harvested approximately 7 to 10 hours into the light period of the day/night cycle, although this timing was not precisely monitored.

RNA was extracted from the plant axes following the protocol of the RNeasy Plant Mini Kit (Qiagen Valencia, CA) and stored at -80ºC prior to shipping to McGill University and Genome Québec Innovation Centre for processing and hybridization to two sets of five Affymetrix GeneChips®(Affymetrix, 2002a), referred to as 22k and 8k arrays, representing 22,810 and 8,297 genes respectively. The protocol of the Genome Centre consisted of 1 µl of 100 pmole/ul T_7-T_{24} primer added to 20 µg of RNA at 2 µg/µl. Superscript II Reverse Transcriptase extends in a 5' to 3' direction from the polyA tail. RNAse H was used to nick the RNA part of the RNA-DNA hybrid; DNA polymerase I created a second strand of cDNA. Double stranded cDNA was transcribed to produce labeled cRNA, which was labeled using biotinylated ribonucleotides.

Fragmentation buffer was used to produce cRNA approximately 100 bp in length. Fifteen µg of the fragmented cRNA was used for each hybridization. Finally, the chips were stained, laser scanned, and analysed (https://genomequebec.mcgill.ca/mpf/info/Old%20labeling%20protocol-last%20version.pdf GenomeQuebec 2004).

Hybridization efficiency of probe sequences can be affected by several factors, including exclusivity of complementarity, sample and probe concentrations, time, temperature, pH, ionic concentration, valency, and density of probes. Affymetrix GeneChips® include *Arabidopsis* maintenance genes GAPDG, ubiquitin, and actin as controls to establish minimal signal thresholds. GeneChips® monitor for efficiency of the photolithographic-generated probes by inclusion of up to 22 replicates of the 25mer oligos. Non-specific hybridization is compensated for by quantification and subtraction of hybridization to probes with a mismatched 13^{th} base. The intensity of hybridization to background noise is compared using a one-sided non-parmetric Wilcoxon Signed Rank Test to calculate *p*-values for the null hypothesis. Detection of hybridization levels are categorized with the flags P(resent), M(arginal) or A(bsent), at a level individually determined for each array. P(resent) signals have greater than 70% detection; A(bsent) hybridization levels are not confidently different from zero; M(arginal) indicates uncertainty. These mechanisms were designed to confer confidence in hybridization specificity and spot scanning sensitivity (Affymetrix, 2002b).

In the present study, five distinct RNA samples were hybridized to GeneChips® representing: three biological replicates of BAP-treated samples (R1, R2, R3) and two controls (C1, C2/T1). C1 represented pooled RNA from controls grown at the same time as BAP-treated populations. The second 'control' chip, C2, was hybridised with RNA from plants not treated with BAP; however, in this case the sample represented the first generation (T_1) descendants of BAP-treated R1. C2 served in statistical analysis of BAP-treated samples, as SAM and Genespring required a second control. C2 was also individually analysed in comparison with C1 for indications of inheritance of altered gene transcript levels, at which times it is referred to as the T1 or C2/T1 chip.

The five samples were hybridized to both 22k and 8k arrays as technical replicates. It was assumed that spot-quality assessment of the intensity of hybridization

by the Genome Québec Innovation Centre was performed accurately. Consistent patterns of induced changes in transcript levels between the 22k and 8k technical replicates increased this confidence.

The 22k GeneChips® were scanned using Microarray Suite5 default settings, followed by interpretation of spot emission and conversion to numerical output by InforSense Kensington Discovery Edition v1.8.2 (KDE®) (www.inforsense.com) software. Hybridization levels were flagged as P(resent), M(arginal) or A(bsent). KDE® performed normalization involving a model-based background correction, quantile normalization at the probe level, and median polish, to produce a robust average of probe intensities for the expression summary.

Data normalization is the first step in numeric manipulation for statistical analysis. The program KDE® performed normalization of elements in the set. Expression values were scaled to 1000 units so that all arrays had the same mean. Application of the formula resulted in a normalization factor (NF) of 1.00168577.

Narray = the total number of elements represented on the arrays

$$NF = \frac{\sum_{i=1}^{Narray} C((C1+C2)/2)}{\sum_{i=1}^{Narray} R((R1+R2+R3)/3)} \qquad (2.1)$$

KDE® calculated ratios between transcript levels of the BAP-treated replicates, R1, R2, and R3 and the control C1, with the lesser number as the divisor so that down-regulated genes were presented as negative numbers rather than decimals. KDE® identified genes with significant change in transcript levels based on comparison of individual BAP-treated replicates with the first control, a ratio or fold change ≥ 2.5, intensity minimum of 200 units, and a flag of P or M for the numerator of the ratio (the denominator could be A, M or P). For the present study, KDE® determined significant changes in transcript levels between the individual BAP-treated replicates, R1,R2, and R3, and the T_1 generation, C2/T1, relative to the control, C1. Conforming to the convention in colour coding of microarray data, red was used to designate a significant

increase in transcript, blue or green a significant decrease, and yellow, black, or no colour for a non-significant change or steady state; purple represented controls.

Analyses of the microarray data were also carried out with the robust criteria of SAM 2.0®, GeneSpring 7.2®, Genesight 4.1®, and Genepublisher 1.03® software. Genesight® and Genepublisher® analyses were interpreted as not useful.

2.3.1.1 Significance Analysis of Microarrays 2.0 (SAM®)

Significance Analysis of Microarrays 2.0 (SAM®) (Tusher et al., 2001), was developed specifically for microarray research and is offered free to academics from Stanford University http://www-stat.stanford.edu/~tibs/SAM/. BAP-treated vs. control microarray data were analysed by SAM® within Microsoft Excel using a modified t-test statistic (known as the S test) that includes a constant in the denominator of the gene-specific t-test to accommodate the greater variability of expression levels sometimes associated with genes expressed at low levels. SAM® uses a non-parametric false discovery rate control method to determine significance (Cui and Churchill, 2003; Storey and Tibshirani, 2003). For this project the three BAP-treated replicates and two controls were subjected to two class, unpaired data analysis.

y_j = 1 or 2; let C_k = [j : y_j = k] for k = 1, 2; let n_k = ≠ of observations in C_k

$$r_i = \bar{x}_{i2} - \bar{x}_{i1}$$
$$s_i = [(1/n_1 + 1/n_2)\{\sum_{j \in C_1}(x_{ij} - \bar{x}_{i1})^2 + \sum_{j \in C_2}(x_{ij} - \bar{x}_{i2})^2\}/(n_1 + n_2 - 2)]^{1/2}$$

$$\text{Let } \bar{x}_{i1} = \sum_{j \in C_1} x_{ij}/n_1, \bar{x}_{i2} = \sum_{j \in C_2} x_{ij}/n_2.$$

$$d = r_i/s_i \qquad (2.2)$$

While there is no 'correct' value for significance parameters, for this study a minimum 2.5-fold change in transcript levels was chosen as significant.

2.3.1.2 GeneSpring 7.2®

GeneSpring 7.2® (Agilent Technologies, Palo Alto, CA, USA; www.agilent.com) was also used to analyse the 22k data. In this case, raw data were entered into the program, which normalized values below 0.01 to 0.01, and each measurement was divided by the 50th percentile of all measurements in that sample. The option to normalize to specific samples was chosen, which measured each gene in each specific sample (R1-3) and divided by the median of that gene's measurements in the corresponding two control samples (C1-2). The treatment parameters were set as R1-3 for BAP-treated and C1-2 for controls with a 2.5-fold threshold for increased and/or decreased expression. GeneSpring® reported from raw data in .txt format identifiers, which were then converted to Atg identifiers with a software tool developed by Dr. N. Provart (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_agi_converter.cgi).

2.3.1.3 Functional Categorization

Gene Ontology (GO) (Berardini et al., 2004) at The *Arabidopsis* Information Resource (TAIR http://www.arabidopsis.org) program was used to categorize significant genes identified by SAM®. Sub-categories of Cellular Components, Molecular Function, and Biological Processes are not mutually exclusive, so GO assigns some genes to more than one category. The percent of significant genes assigned to categories in BAP-treated samples were compared to the total data analysed with SAM.

2.3.1.4 Mining the Data

As well as the analysis of the microarrays by computer programs to identify genes with statistically significant changes in transcript levels, specific genes of interest were mined from the data based on locus identifiers listed at TAIR.

2.3.1.5 Microarray Database

Tools within the public access microarray database Genevestigator (https://www.genevestigator.ethz.ch.) (Zimmermann et al., 2004) were used to compare the data of the present study with other microarray experiments. Gene-Chronologer was used as an indicator of control transcript levels of specific genes during development.

Meta-Analyzer was used to examine expression profiles of genes responding to various plant hormones (primarily based on microarray data from Yoshida's Laboratory, Saitama, Japan) and stress conditions.

2.3.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) provides a non-hybridization-based method of confirming and quantifying transcriptional regulation of specific genes identified by microarrays. This is a valuable technology for determining changes in expression over developmental time, as well as the study of genes with exceptionally low transcript levels, such as transcription factors.

The main axes of 50 plants, including the root and shoot apical meristems, with leaves removed, were collected and RNA isolated (section 2.3.1) at 4, 24, 48, 96, and 192 h after BAP-treatment at the 4-5 leaf stage. Equivalent controls were also harvested and RNA isolated. Time courses of three separate populations of both control and BAP-treated plants were collected; in each case plants were also retained for phenotype analysis. RT-PCR on total RNA was carried out using the OneStep RT-PCR Kit (Qiagen Valencia, CA).

18S ribosomal RNA was the internal control. 18S competimers are primers with the same sequences as 18S-5' and 18S-3' but with a dideoxythymidine at the 3' end (18S sequences supplied by Kaplan and Guy, U of F, Gainesville, Florida, USA) to restrict polymerase activity and slow replication to avert band saturation. The appropriate 18S primer:competimer ratio was optimized to reduce the 18S signal in order to generate unsaturated RT-PCR signals for the RNA concentrations of the gene specific products produced by gene specific primers (Sung et al., 2001) (Table 2.1). This optimization identified 64 ng RNA, 1:6 ratio of 18S primers:competimers; 25 PCR cycles for the amplification of *CLV1*, *CLV1-LIKE*, and *GH3-12* transcripts, and 37 PCR cycles for the amplification of *WUS* and *AP1* transcripts. Transcript amplified by RT-PCR was analysed on ethidium-bromide stained gels using Gel Doc 2000 Quantity One software. Ratios of the gene of interest to the 18S internal control were calculated and graphed with standard error bars. Statistical analysis was carried out with SAS version 8.2 for Windows (SAS Institute Inc. Cary, NC, USA). Differences were considered significant at $p \leq 0.05$.

Table 2.1. Gene specific primers

5' Primer	**3' Primer**
18S rRNA	
GGAGCGATTTGTCTGGTT	TGATGACTCGCGCTTACT
AP1 At1g69120	
AAGGATCAAAAATGGGAAGG	AGTGCGGATGTGCTTAAGAG
CLV1 At1g75820	
CTGCTTCTGAGTGTATGTCTTC	CGGATTTAGGAGGGTTAGTGAG
CLV1-LIKE At1g08590	
GGGCTTGTGTTATCTTCACCATG	CACGGATTTAGGAGGGTTAGTGAG
GH3-12 At5g13320	
AGTCGCAACCAGCAGCTATT	CCATTGAACCAGTGACAACG
WUS At2g17950	
TTCAACGGAACAAACATGAC	GTGCATAGGGAAGAGAGGAA

Gene specific primers used for RT-PCR analysis of transcript abundance.

3. RESULTS

3.1 Microarray Data Analysis

The use of microarray technology in biological studies has grown exponentially in the last decade resulting in overwhelming quantities of data, yet consistent guidelines in experiment design and data manipulation are still lacking (Provart and McCourt, 2004). In contrast to projects striving to generate data, the challenge with transcriptome studies is interpreting and representing the massive data in meaningful ways. The microarray data in this study, supplemented by other methods of investigation, were concerned with three broad categories: BAP-altered phenotypes; cytokinin action in relation to responses to the environment, associated regulatory hormone crosstalk, and signal transduction; epigenetic inheritance. Determining criteria for sorting the 22,810 data points required flexibility and revision. In this study, three computer programs were utilized to statistically analyse the data. Mining of individual genes and intuitive interpretation of expression patterns also proved essential.

3.1.1 Determination of Significant Genes

KDE® was the initial program used to read the microarray spots. The percentage of spots with P(resent) levels of hybridization for each array: C1 62.9%, C2/T1 63.3%, R1 63.9%, R2 65.5%, and R3 66.6%. KDE® was used to identify significant responses to BAP in the biological replicates (R1, R2, R3) and the next generation sample (C2/T1), relative to the primary control (C1) (Table 3.1). The ratio of change in transcript levels determined by KDE® was used for comparison of individual arrays.

The computer program SAM® (Tusher et al., 2001) was used for statistical analysis of the three BAP-treated replicates and two controls. The data were sorted for relevance and flags before analysis by SAM®. The 64 genes with null identity were removed, as these genes could not be investigated further. Also, 7802 genes with A(bsent) hybridisation signal in 3-5 of the 5 samples were eliminated; 1703 genes with a reading of A(bsent) in one or two of the five samples were retained. Also, the 74 genes with hybridisation signals M(arginal) or P(resent) in C1 and C2/T1, but A(bsent) in the 3

Table 3.1. Computer program analyses of microarray data

Program	GeneChip®	Significant Genes	Increased Transcript	Decreased Transcript
KDE®				
	22k R1	695	499 (72%)	196 (28%)
	22k R2	560	421 (75%)	139 (25%)
	22k R3	1,765	1050 (59%)	715 (41%)
	22k C2/T1	247	68 (28%)	179 (72%)
	22k R1-3	334	288 (87%)	43 (13%)
SAM®				
	22k R1-3	653	591 (90%)	62 (10%)
	8k R1-3	293	267 (91%)	26 (9%)
GeneSpring®				
	22k R1-3	1,199	900 (75%)	299 (25%)

Computer programs KDE®, SAM®, and GeneSpring® were used to analyse 22k and/or 8k microarray data to determine significant BAP-induced changes in transcript levels. KDE® determined significance by comparison of transcript levels in individual BAP-treated replicates (R1-R3) and the next generation T1, relative to the C1 control. SAM® and GeneSpring® determined significance in the combined BAP-treated replicates (R1-R3) compared to the two controls (C1 and C2). The percentages of the total number of significant genes with increased or decreased levels are also listed.

BAP-treated replicates were retained, as these were interpreted as potential candidates for significant downregulation. These criteria determined 14,944 genes of interest from the 22,810 genes represented on the 22k chips and 4,843 genes from the 8k chips.

Two class, unpaired data analysis was performed by SAM®. For the 22k data, SAM® identified 5,554 significant genes, 4,319 (78%) with increased and 1,235 (22%) with decreased transcript levels. Further sorting by implementing a threshold of 2.5-fold change in transcript levels identified 653 significant genes, of which, 591 (90%) had increased and 62 (10%) decreased transcript levels (Table 3.1). Throughout this dissertation, data analysis concerned with functional categorization, chromosomal distribution, and references to 'significant' genes in BAP-treated samples, feature these 653 genes. Similar criteria were used for the 8k data, where SAM® recognized 2,422 significant genes. Implementation of a 2.5 threshold determined 293 genes (Table 3.1). 60% of the 293 significant genes from the 8k arrays were also significant on the 22k. Some genes spotted on the 8k array were not represented on the 22k.

Filtering for flags is a tool built into the GeneSpring® 7.2 microarray analysis program to facilitate adjustment of stringency. P(resent) or M(arginal) in 3 of 5 of the 22k chips was chosen for this study. GeneSpring® identified 1,199 genes with significant change in transcript levels in BAP-treated samples relative to controls (Table 3.1). The 8k data were not analysed with GeneSpring®.

Throughout the results section, specific genes of interest are presented in tables that include the microarray hybridization values, ratio differences between BAP-treated replicates and the first control, and denotation of significance, as determined by SAM® and GeneSpring® (Table 3.2). The range of significant genes determined by each of the computer programs was divided into thirds. The most significant third is denoted by '***', the next by '**', and the last by '*'. The top fifteen genes with increased transcript levels from the 22k data, as determined by the three computer programs, were compared (Table 3.3). The identity/function of the genes was found at TAIR.

Table 3.2. Range of significance determined by SAM® and GeneSpring®

GeneChips®	**22k & 8k**	**22k & 8k**	**22k**	**Tables**
Statistical Program	SAM® d-value	SAM® d-value	GeneSpring® t-test p-value	
BAP-induced changes	Increased transcript levels	Decreased transcript levels	Increased and decreased levels	Denoted in tables:
	$18.0 \geq x \geq 3.0$	$-4.0 \geq x \geq -15.0$	$0.01 >$	***
	$3.0 > x \geq 2.0$	$-2.5 > x \geq -4.0$	$0.1 > x \geq 0.01$	**
	$2.0 > x \geq 1.0$	$-2.0 > x \geq -2.5$	$0.5 \geq x \geq 0.1$	*

Throughout the results, the significant BAP-treated genes determined by SAM® and GeneSpring® were divided into thirds and these categories are denoted by '*' (significant), '**' (more significant), or '***' (most significant) in microarray data tables.

Table 3.3. Top 15 genes with increased transcript levels

KDE		**SAM**		**GeneSpring**	
At5g13320	auxin-resp. GH3 family	At3g56400	*WRKY70*	At5g22570	*WRKY38*
At1g26420	monooxygenase activity	**At4g33050**	**calmodulin-binding**	**At3g17690**	**nucleotide binding**
At1g33960	AIG1	At2g26400	iron-deficiency	**At5g24210**	**lipase class 3 family**
At1g09080	binding protein 3 (BiP-3)	At1g21270	*WAK2*	At4g23320	protein kinase
At1g10585	bHLH transcription factor	At2g41410	calmodulin-like protein	**At3g57950**	**unknown protein**
At2g30770	putative cytochrome P450	**At5g24210**	**lipase class 3 family**	**At4g33050**	**calmodulin-binding**
At3g28210	zinc finger protein	**At3g56710**	***SIB1* light-dependent**	At1g68765	unknown protein
At1g57650	disease resistance protein	At3g29240	thylakoid lumen	**At1g16260**	**protein kinase**
At5g02780	unknown protein	**At3g17690**	**nucleotide binding**	At3g28580	ATPase family protein
At4g23150	protein kinase family	**At1g51920**	**unknown protein**	**At1g51920**	**unknown protein**
At2g29470	*GST21*	At2g46400	*WRKY46*	At4g38560	unknown protein
At3g21080	ABC transporter-related	At4g26090	*RSP2*, disease resistance	At4g02410	protein kinase
At2g38340	ERF/AP2 transc. factor	**At1g16260**	**protein kinase**	**At3g56710**	***SIB1* light-dependent**
At3g57950	**unknown protein**	At5g25440	protein kinase	At5g24240	protein kinase
At1g01480	unknown protein	At1g22400	glucosyl transferase	At1g65500	unknown protein

The 15 genes identified by the computer programs KDE, SAM, and GeneSpring as the most significant BAP-induced increases in transcript levels relative to controls, in the 22k data. Genes common to more than one column are bolded.

3.1.2 Categorization of Significant Genes

The mandate of Gene Ontology (GO http://www.geneontology.org/GO) is to develop consistent terminology to describe gene products in terms of their associated cellular components, molecular functions, and biological processes, in a species-independent manner. The 653 significant genes identified by SAM® were categorized using the GO program available at TAIR (Figures 3.1a, 3.2a, 3.3a) as were the 14,944 genes analysed by SAM® (Figures 3.1b, 3.2b, 3.3b). Sub-categories of genes emphasized and de-emphasized by BAP treatment were determined after the data were normalized to the frequency of the initial 14,944 genes in each category. A 2-fold preferential emphasis or de-emphasis of a sub-category for the mean of the three replicates was noted (Figure 3.4). The categories emphasized in each of the three individual biological replicates (R1-3) were also determined based on significant genes identified by KDE® (Figure 3.5). Abbreviated definitions of the GO categories showing a) emphasis or b) de-emphasis by approximately 2-fold in BAP-treated samples (Figures 3.1, 3.2, 3.3) are listed; full definitions can be found at TAIR.

3.1.2.1 Definitions of GO Categories

a) emphasized

Cell Wall – the rigid or semi-rigid envelope lying outside the cell membrane of plant
Kinase Activity – catalysis of the transfer of a phosphate group to a substrate molecule
Transcription Factor Activity – a protein required to initiate or regulate transcription
Receptor Binding or Activity – growth factor stimulating a cell to grow or proliferate; hormone activity; transmembrane receptor kinase activity
Response to Abiotic or Biotic Stimulus – response to stimuli, including, but not limited to: heat, salinity, cold, UV, light, toxins, desiccation, foreign bodies, and injury
Response to Stress – a change in state or activity of an organism or cell (in terms of movement, secretion, enzyme production, gene expression, etc.) that occurs in response to stress, usually, but not necessarily exogenous

b) de-emphasised

Ribosome – the site of protein biosynthesis
Plastid – outer or inner chloroplast membrane
Structural Molecule Activity – molecule that contributes to the structural integrity of a complex or assembly within or outside a cell
Nucleic Acid Binding – interacting selectively with any nucleic acid
Cell Organization – multidimensional cell growth
DNA or RNA metabolism – the processes of restoring DNA after damage; synthesizing new strands of DNA; transfer of a methyl group to adenine or cytosine

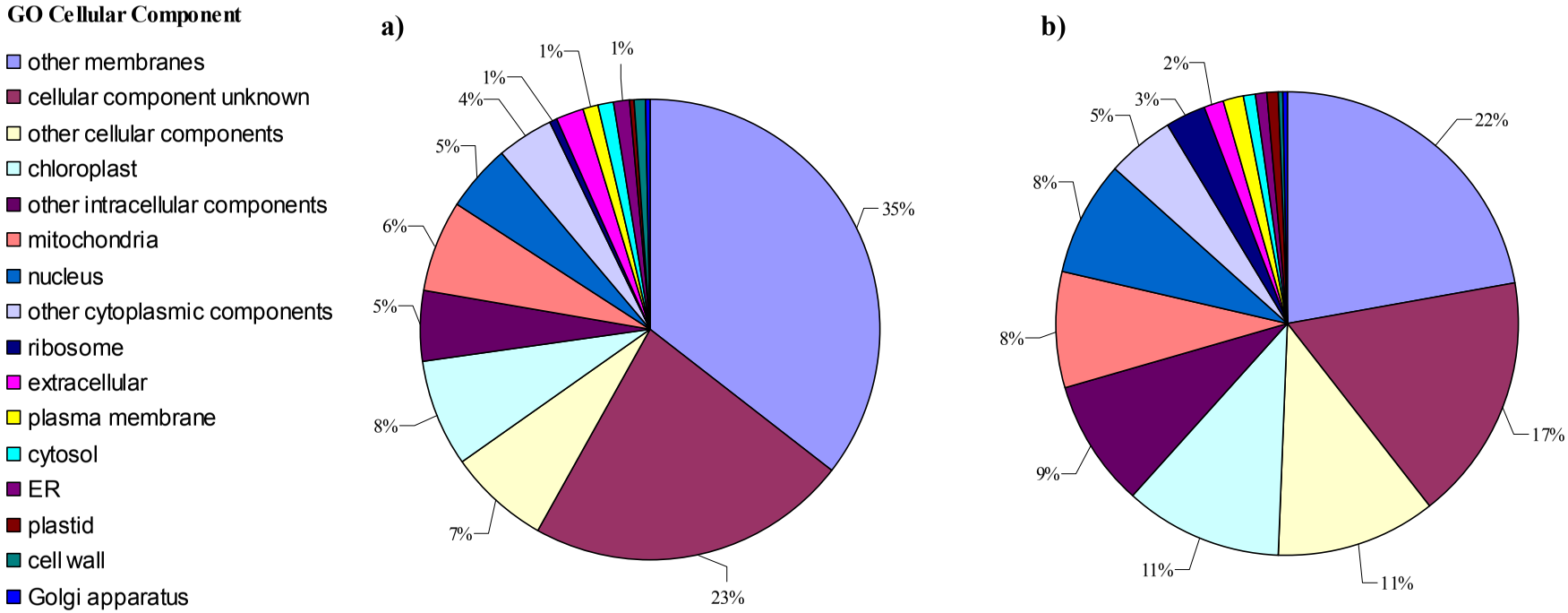


Figure 3.1. GO categorization of the 653 significant genes from BAP-treated samples identified by SAM (a) relative to the categorization of the 14,944 genes that were analysed by SAM (b) in sub-categories of cellular components.

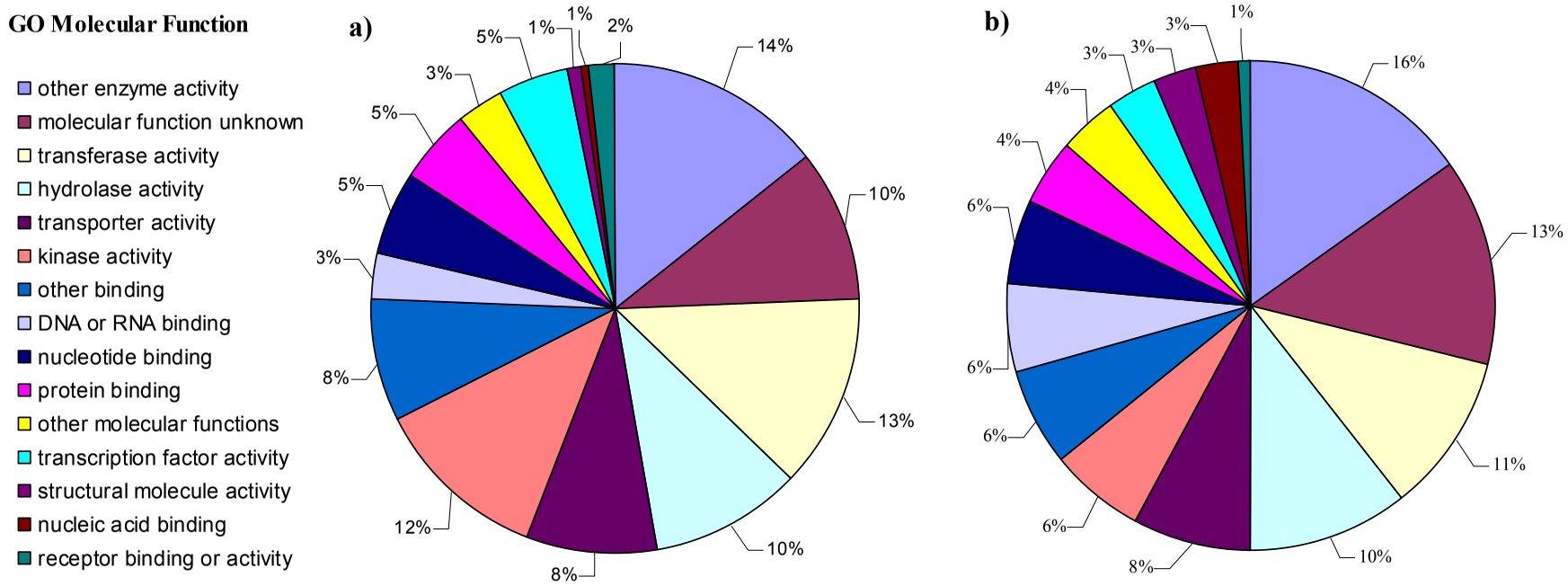


Figure 3.2. GO categorization of the 653 significant genes from BAP-treated samples identified by SAM (a) relative to the categorization of the 14,944 genes that were analysed by SAM (b) in sub-categories of molecular function.

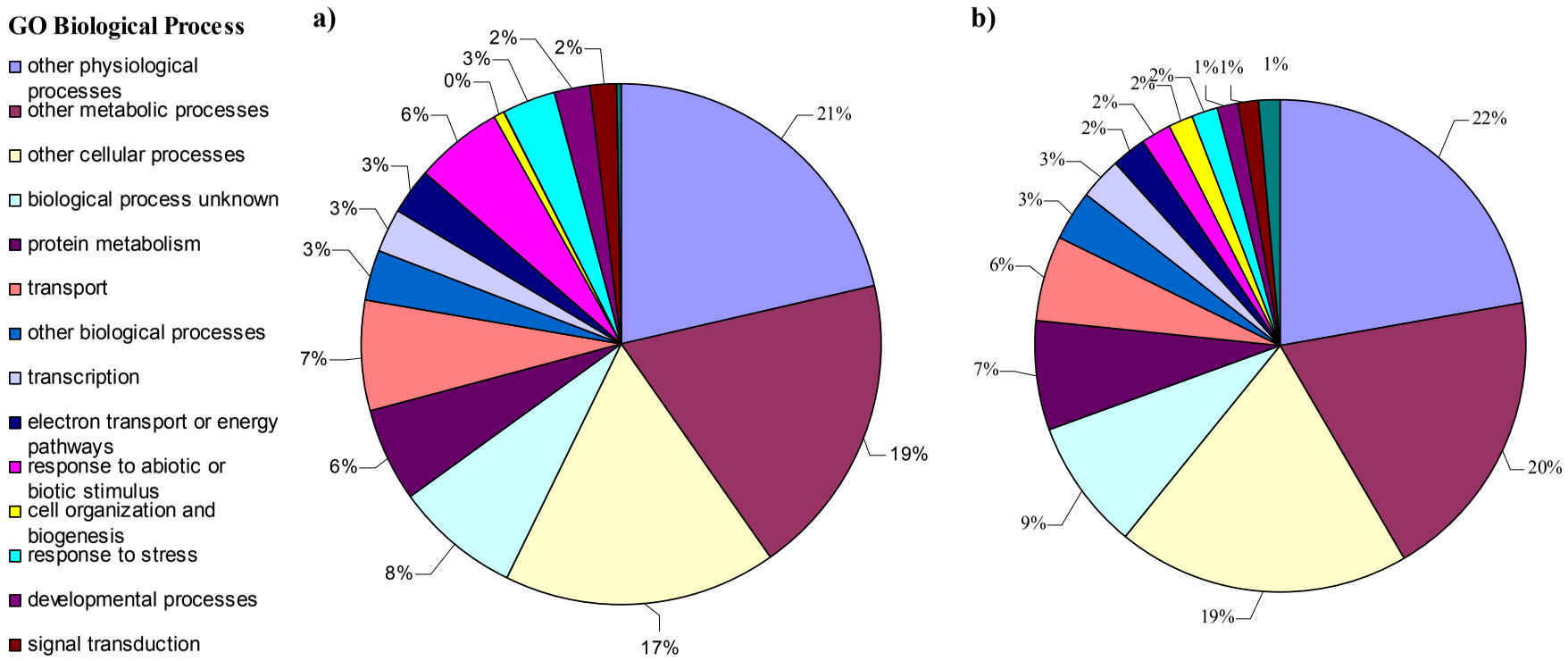


Figure 3.3. GO categorization of the 653 significant genes from BAP-treated samples identified by SAM (a) relative to the categorization of the 14,944 genes that were analysed by SAM (b) in sub-categories of biological processes.

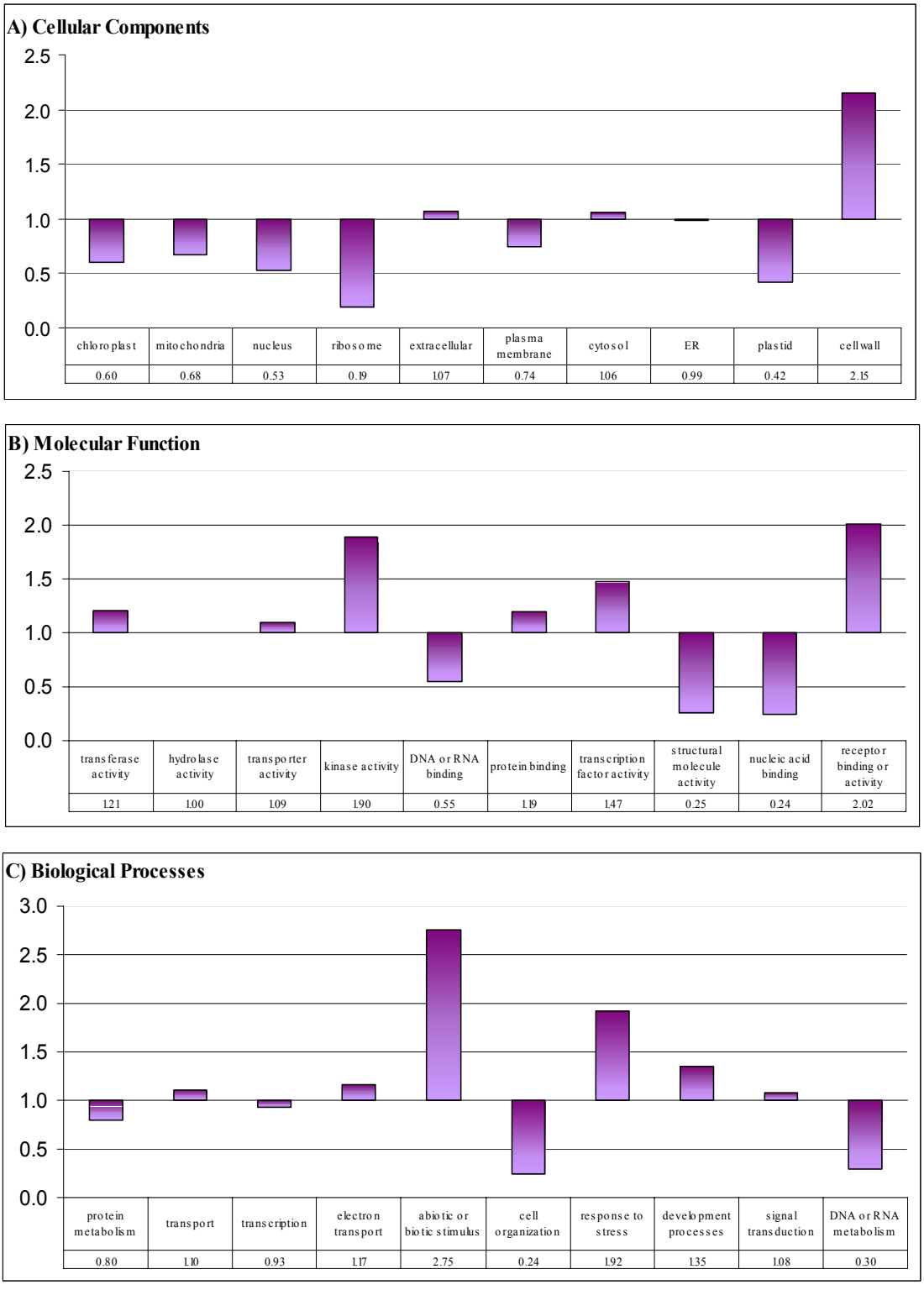
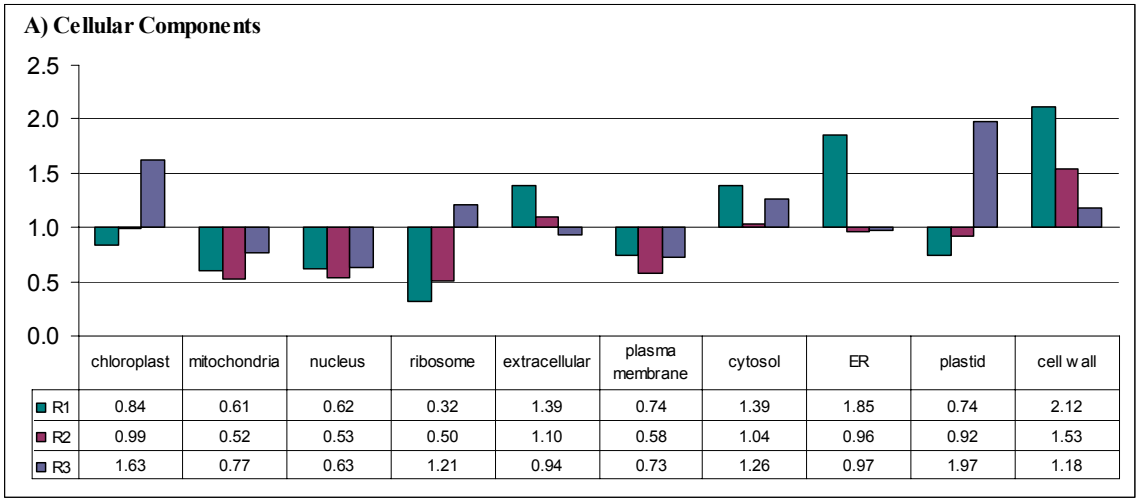
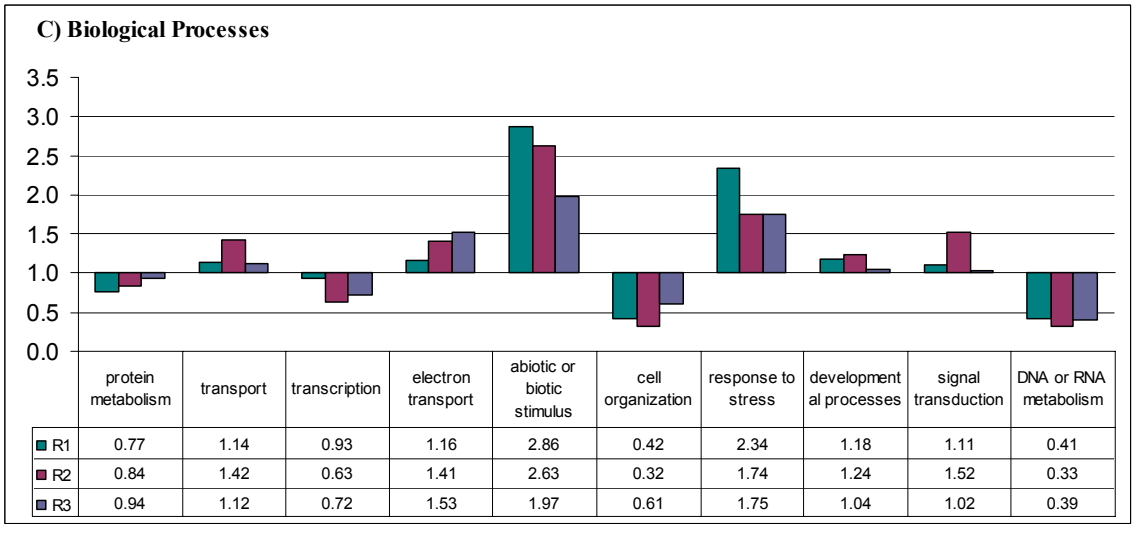


Figure 3.4. GO categorization of the 653 significant genes, as determined by SAM®, from BAP-treated samples. The y-axis represents frequency of significant genes in each category normalized to the frequency of all genes of the analysed data in each category.



	chloroplast	mitochondria	nucleus	ribosome	extracellular	plasma membrane	cytosol	ER	plastid	cell wall
R1	0.84	0.61	0.62	0.32	1.39	0.74	1.39	1.85	0.74	2.12
R2	0.99	0.52	0.53	0.50	1.10	0.58	1.04	0.96	0.92	1.53
R3	1.63	0.77	0.63	1.21	0.94	0.73	1.26	0.97	1.97	1.18

	transferase activity	hydrolase activity	transporter activity	kinase activity	DNA or RNA binding	protein binding	transcription factor activity	structural molecule activity	nucleic acid binding	receptor binding or activity
R1	1.02	1.03	1.18	1.19	0.50	1.30	1.17	0.44	0.15	0.90
R2	1.20	0.93	1.26	1.71	0.38	1.12	1.00	0.35	0.22	1.58
R3	1.01	1.08	1.24	1.18	0.59	1.10	0.96	1.02	0.39	0.98



	protein metabolism	transport	transcription	electron transport	abiotic or biotic stimulus	cell organization	response to stress	developmental processes	signal transduction	DNA or RNA metabolism
R1	0.77	1.14	0.93	1.16	2.86	0.42	2.34	1.18	1.11	0.41
R2	0.84	1.42	0.63	1.41	2.63	0.32	1.74	1.24	1.52	0.33
R3	0.94	1.12	0.72	1.53	1.97	0.61	1.75	1.04	1.02	0.39

Figure 3.5. GO categorization of the significant genes, as determined by KDE in each of the BAP-treated replicates (R1-3). The y-axis represents frequency of significant genes in each category normalized to frequency of all genes on the 22k array in each category.

3.2 BAP-altered Development

Cytokinins play a role in a wide array of important biological processes, including chloroplast development, apical dominance, flowering, and delay of senescence (Mok, 1994); therefore, the *Arabidopsis* plants treated with BAP were examined for altered phenotypes.

3.2.1 Floral Phenotypes

Flower development in *Arabidopsis* follows a conserved pattern of four whorls: a calyx of four sepals, a corolla of four petals, four long medial and two short lateral stamens, and congenitally fused carpels forming a double-locule gynoecium with a false septum (Figure 3.6a) (Smyth et al., 1990). Aberrant floral morphology was observed in 1.8% of flowers in the first five flower positions of control plants (Table 3.4), while 82% of the BAP-treated plants produced at least one aberrant flower, with an average of 50% of the flowers in the first five flower positions showing aberrant development; 30% of these featured a single aberrant phenotype and 70% multiple aberrant characters.

The most common BAP-induced aberrant floral phenotype was an increase in organ number. In control populations, of 1500 flowers examined from the first 15 positions on the racemes of 100 plants, less than 2% showed an increase in floral organ number. Comparatively, 81% of 100 BAP-treated plants produced at least one flower with increased organ number; of the responsive plants, 36% of the flowers from the proximal 15 flower positions showed an increase in organ number, with the occurrence decreasing acropetally (Figure 3.7). The BAP-treated floral phenotype (Figure 3.6b) resembled *clv1* mutants (Figure 3.6c), with a similar, but somewhat lower average organ number than the strong *clv1-4* mutant (Table 3.5). When the fourth whorl was affected, increased carpel number resulted in siliques resembling a *clv1* phenotype (Figure 3.6e-f).

Increased meristem size or activity in BAP-treated plants was indicated by substantially greater diameters of the raceme rachis than control and *clv1-1* plants. The diameter of transverse sections of the rachis, between the first and second flower positions, was, on average: controls 0.78 ± 0.02 mm (Figure 3.8a), BAP-treated 1.03 ± 0.03 mm (Figure 3.8b), and *clv1-1* 0.84 ± 0.01 (Figure 3.8c).

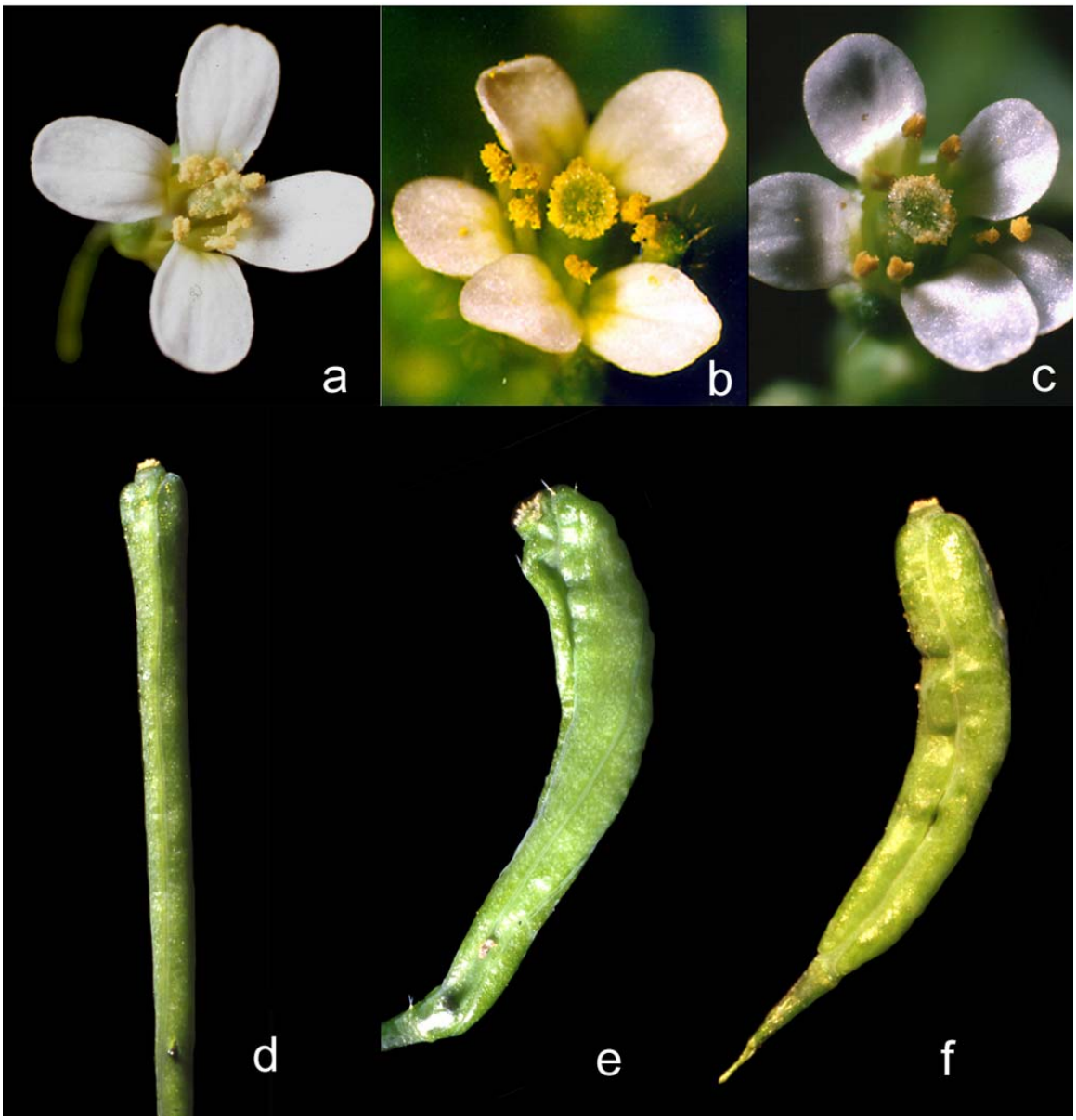
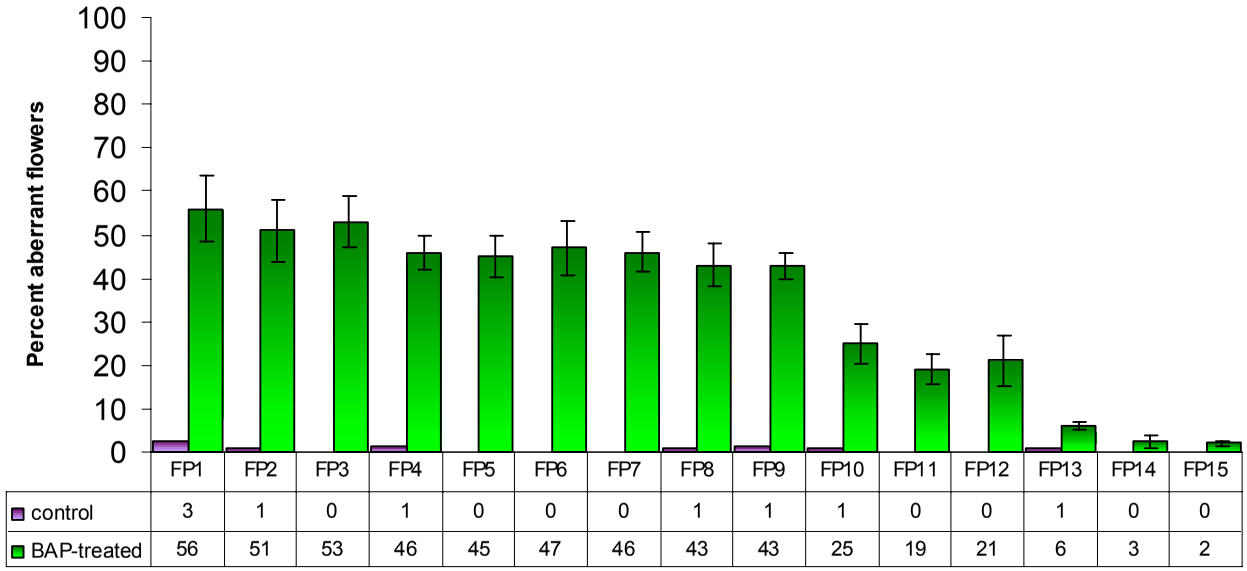


Figure 3.6. Comparison of flowers and siliques. (a and d) *Arabidopsis thaliana* Landsberg *erecta* controls; (b and e) BAP-treated wild type; (c and f) *clv1-1* mutant.

Table 3.4. Aberrant flower development in control (C1-2) and BAP-treated populations (R1-3) harvested for microarray analysis.

Chip	N	Af	↑s	↓s	↑p	↓p	↑st	fst	pst	↑c	ectopic bud	abd
C1	685	1.8	0.6	0.0	0.6	0.0	0.5	0.1	0.0	0.0	0.0	0.0
C2	205	13.1	2.0	1.2	3.6	1.0	1.4	2.3	0.5	1.8	0.0	0.0
R1	440	41.3	21.3	2.2	23.3	3.4	17.2	1.3	1.4	5.2	0.4	0.0
R2	425	53.2	15.2	2.5	23.4	1.1	13.7	0.5	1.1	3.4	0.0	0.0
R3	245	56.3	17.5	2.0	24.5	1.2	14.3	0.5	1.2	11.8	0.8	13.0
Mean*		**50.3 ±4.6**	**18.0 ±1.8**	**2.2 ±0.2**	**23.7 ±0.4**	**1.9 ±0.8**	**15.1 ±1.1**	**0.8 ±0.3**	**1.2 ±0.1**	**6.8 ±2.4**	**0.4 ±0.2**	**4.3 ±4.1**

*Means are percentages of aberrant phenotypes in BAP-treated replicates R1-3.
N = number of flowers (first five flower positions per plant); af=aberrant flowers; ↑=increase in organ number; ↓=decrease in organ number; s=sepals; p=petals; st=stamens; fst=forked stamen; pst=petaloid stamen; c=carpels; abd=arrested bud development.
C2 also represents the T_1 generation of BAP-treated R1 plants. Data in percentages.



	FP1	FP2	FP3	FP4	FP5	FP6	FP7	FP8	FP9	FP10	FP11	FP12	FP13	FP14	FP15
control	3	1	0	1	0	0	0	1	1	1	0	0	1	0	0
BAP-treated	56	51	53	46	45	47	46	43	43	25	19	21	6	3	2

Figure 3.7. Percent of flowers showing increased organ phenotype, by flower position (FP) in control and BAP-treated racemes. N = control, 144; BAP-treated, 217 plants.

Table 3.5. The average number of organs in the first five flower positions of the raceme.

	Sepals	**Petals**	**Stamens**	**Carpels**
BAP-treated	4.37 ± 0.1	4.52 ± 0.1	6.39 ± 0.1	2.72 ± 0.1
clv1-4	4.9 ± 0.7	4.6 ± 0.7	9.3 ± 1.0	4.5 ± 0.9

Comparison of organ number in BAP-treated (N = 75) and *clv1-4* (N = 10) *Arabidopsis*. Data for *clv1-4* from (Schoof et al., 2000).

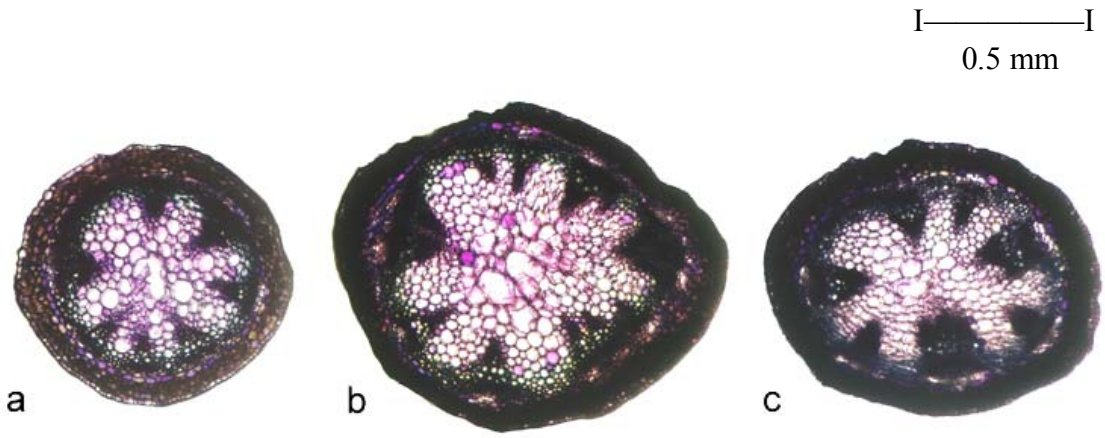


Figure 3.8. Rachis transverse-sections: (a) control (b) BAP-treated wild type (c) *clv1-1*.

The size of the floral meristem has previously been correlated to the number of organ primordia produced (Clark et al., 1993). The apices of BAP-treated plants were compared to those of controls and *clv1-4* mutants with SEM (Figure 3.9). Although a slight increase in size was perceived in treated plants, significant replicates were not found. This may reflect the fact that not all plants responded to the BAP treatment, or that an increase in apical cells occurred after the stage of development analysed. To facilitate correlation of meristem size to floral organ number, dental-style impressions were made of the shoot apex of living plants to count apical cells from the time of BAP treatment through flowering in individual plants. Unfortunately the technique failed due to critical damage incurred in attempting to remove the plethora of trichomes obstructing access to the minute meristem surfaces.

Besides the floral phenotype of increased organ number, BAP induced a reduction in organ number in some flowers (Table 3.4). A common, naturally occurring variation in *Arabidopsis* is a reduction in the number of stamens in up to 25% of flowers (Smyth et al., 1990); therefore, the decreased-stamen phenotype was not included in control or BAP-induced floral phenotypes. Other aberrant phenotypes induced by BAP included buds in the axils of sepals (Figure 3.10a), stamen filament dichotomy or trichotomy, (Figure 3.10b), petaloid-stamen intermediary organs (Figure 3.10c), trichomes on floral organs other than sepals, e.g., gynoecium (Figure 3.10c), a protruding false septum, most often in ovaries with three or four carpels (Figure 3.10d), and arrested bud development (Figure 3.10e). In the third BAP-treated replicate, 13% of the flowers showed arrested bud development (Table 3.4).

3.2.2 Other Phenotypes

Although not included in aberrant flower data, the BAP treatment often increased sepal trichome number from about 0-10 in controls to 10-40 in treated plants. The trichomes on *Arabidopsis* sepals are usually simple, non-branched (Figure 3.10f); approximately 35% of trichomes on sepals of BAP-treated flowers were stalked and dichotomously forked (Figure 3.10g), as were those occasionally produced on the gynoecium (Figure 3.10c). In addition, BAP-treated plants showed robust vegetative growth, increased lateral shoot development and elongation, i.e. reduced apical

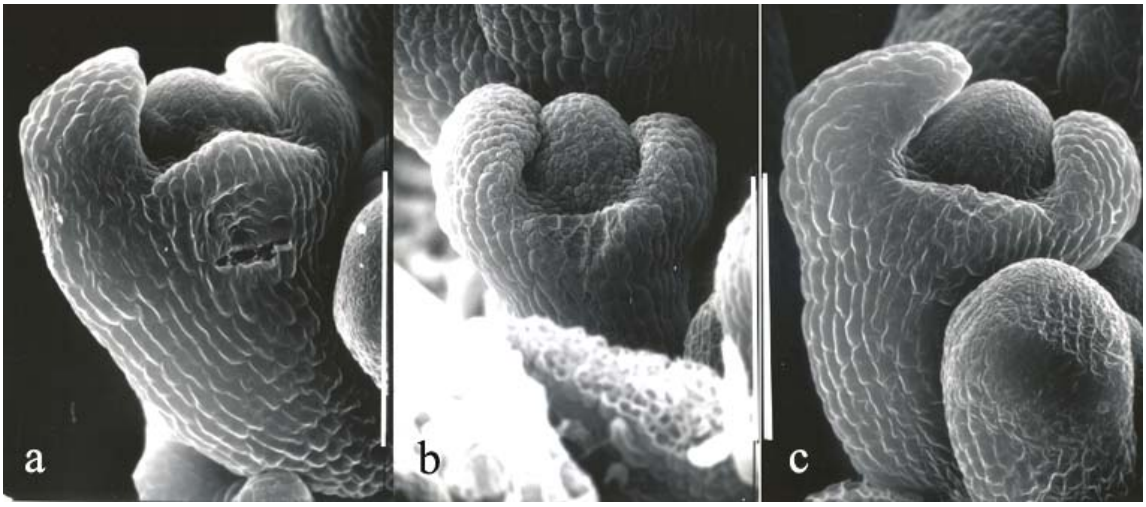


Figure 3.9. Floral meristems with sepal primordia: (a) control, (b) BAP-treated wild - type, and (c) *clv1-4*. Scale bars = 0.1 mm.

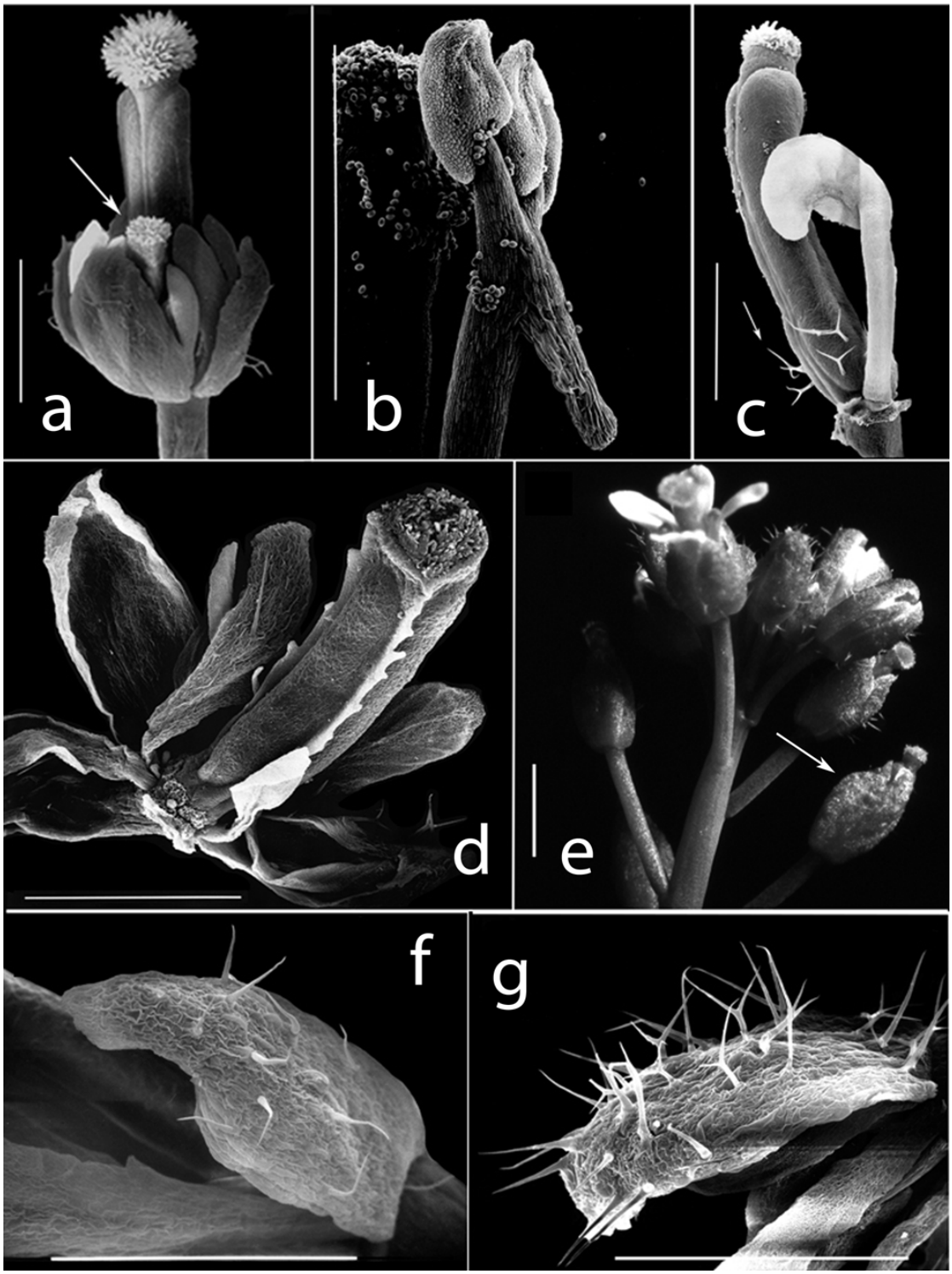


Figure 3.10. BAP-treatment floral phenotypes. (a) Bud in the axil of a sepal (arrow). (b) Stamen trichotomy; two forks bearing anthers and a third reflexed filamentous structure (T_1 plant). (c) Petaloid-stamen and trichomes on gynoecium (arrow). (d) Increased carpel number and protruding ridged false septa. (e) Arrested bud development (arrow). (f) Simple trichomes on sepal of a control flower and (g) forked trichomes on a sepal of a BAP-treated flower. All scale bars = 1 mm.

dominance, dark green leaves (some with a purple tinge), branched rooting, and delayed senescence.

Within 24 hours of treatment, some populations showed a hypersensitive response to BAP, with up to ¼ of the young plants having a cotyledon or leaf turn tan-colour, but not wither. A few plants died. Less plants turned tan or died if the pots were watered and covered with a clear lid following BAP treatment.

3.3 BAP-induced Transcriptome Changes Linked to Phenotype

3.3.1 Increased Floral Organ Number and Shoot Meristem

Based on the phenotypic similarity of BAP-treated plants to *clv* mutants, members of the well-characterized *CLV1* pathway, regulating organ initiation and meristematic cell proliferation (Clark et al., 1997), were mined from the data (Table 3.6). Of these genes, *CLV1* transcript levels were significantly decreased by BAP treatment.

3.3.1.1 *WUS* Transcript Levels in Microarray Samples

As microarray hybridization of *WUS* was flagged A(bsent), RT-PCR, a more sensitive technique for measuring transcript levels, was carried out with the RNA samples used in the microarray analysis. *WUS* transcript levels were higher than the control in all BAP-treated replicates while the T_1 generation equaled the control (Figure 3.11a). The mean of the RT-PCR measurement of the BAP-treated replicates divided by the control indicated a 2.36-fold increase in transcript (Figure 3.11b), compared to an average 2.28 increase for the microarray values (Table 3.6).

3.3.1.2 *CLV1* and *WUS* Transcript Levels During Flowering

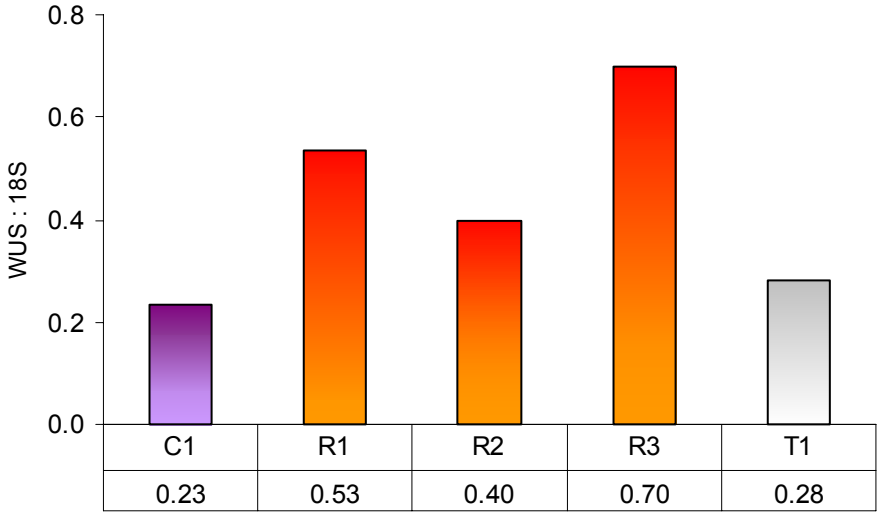
RT-PCR was used to compare *CLV1* (Figure 3.12a) and *WUS* (Figure 3.12b) transcript abundance in BAP-treated and control populations over an 8-day post-treatment period (4, 24, 48, 96, 192 hr). Four hours after treatment, transcript abundance of *CLV1* was lower than controls ($p = 0.0835$), and at 24 and 48 h was significantly lower ($p = 0.0447$; 0.0086 respectively). *WUS* showed significantly higher transcript

Table 3.6. Microarray data of genes regulating the *CLAVATA1* pathway

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***CLAVATA 1* (*CLV1*) At1g75820**												
22k	P	P	P	P	P	3446.95	1698.47	918.27	1067.44	626.83		
								-3.75	-3.23	-5.50	*	**
8k	P	P	P	P	P	525.48	362.68	108.30	267.49	176.07		
								-4.85	-1.96	-2.98	--	
***CLV2* At1g65380**												
22k	P	P	P	P	P	459.39	426.03	520.33	411.46	442.89		
								1.13	-1.12	-1.04	--	--
8k	P	P	P	P	P	367.29	163.21	47.97	176.86	143.16		
								-7.66	-2.08	-2.57	--	
***CLV3* At2g27250**												
22k	A	A	A	A	A	16.79	15.08	12.01	4.43	32.07		
								-1.40	-3.79	1.91	--	--
***KINASE ASSOCIATED PROTEIN PHOSPHATASE* (*KAPP*) At5g19280**												
22k	P	P	P	P	P	354.84	256.37	400.20	369.36	376.34		
								1.13	1.04	1.06	--	--
8k	P	P	P	P	P	1303.50	1347.63	2307.65	2111.95	2578.54		
								1.77	1.62	1.98	--	
***POLTERGEIST* (*POL*) At2g46920**												
22k	P	P	P	P	P	757.76	856.77	876.98	971.40	951.74		
								1.16	1.28	1.26	--	--
***SHEPHERD* (*SHD*) At4g24190**												
22k	P	P	P	P	P	9917.33	12289.84	19457.14	15892.62	21686.67		
								1.96	1.60	2.19	--	--
8k	P	P	P	P	P	7881.45	7162.89	12928.66	10951.78	16233.96		
								1.64	1.39	2.06	--	
8k	P	P	P	P	P	3437.31	4224.24	8053.16	5907.71	10714.03		
								2.34	1.72	3.12	--	
***HISTONE ACETYLTRANSFERASE 1* (*GNC5*) At3g54610**												
22k	A	A	A	M	A	336.53	352.51	312.35	322.82	421.11		
								-1.08	-1.04	1.25	--	--
***WUSCHEL* (*WUS*) At2g17950**												
22k	A	A	A	A	A	16.79	86.71	40.55	51.71	22.39		
								2.42	3.08	1.33	--	--
8k	A	A	A	A	A	25.80	4.77	8.00	34.63	9.87		
								-3.23	1.34	-2.61	--	

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; blue is decreased transcript levels.

a)



b)

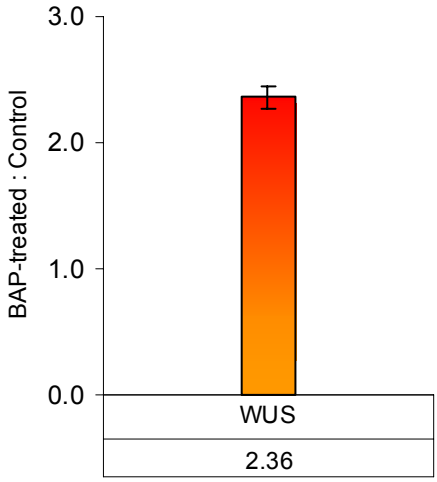


Figure 3.11. RT-PCR amplification of *WUS* transcript levels in (a) microarray samples. Populations harvested 48 h after treatment: control (C1), BAP-treated replicates (R1-3), and second non-treated control (C2 also represents T_1). (b) Mean of transcript level increase R1-3 relative to C1 microarray data.

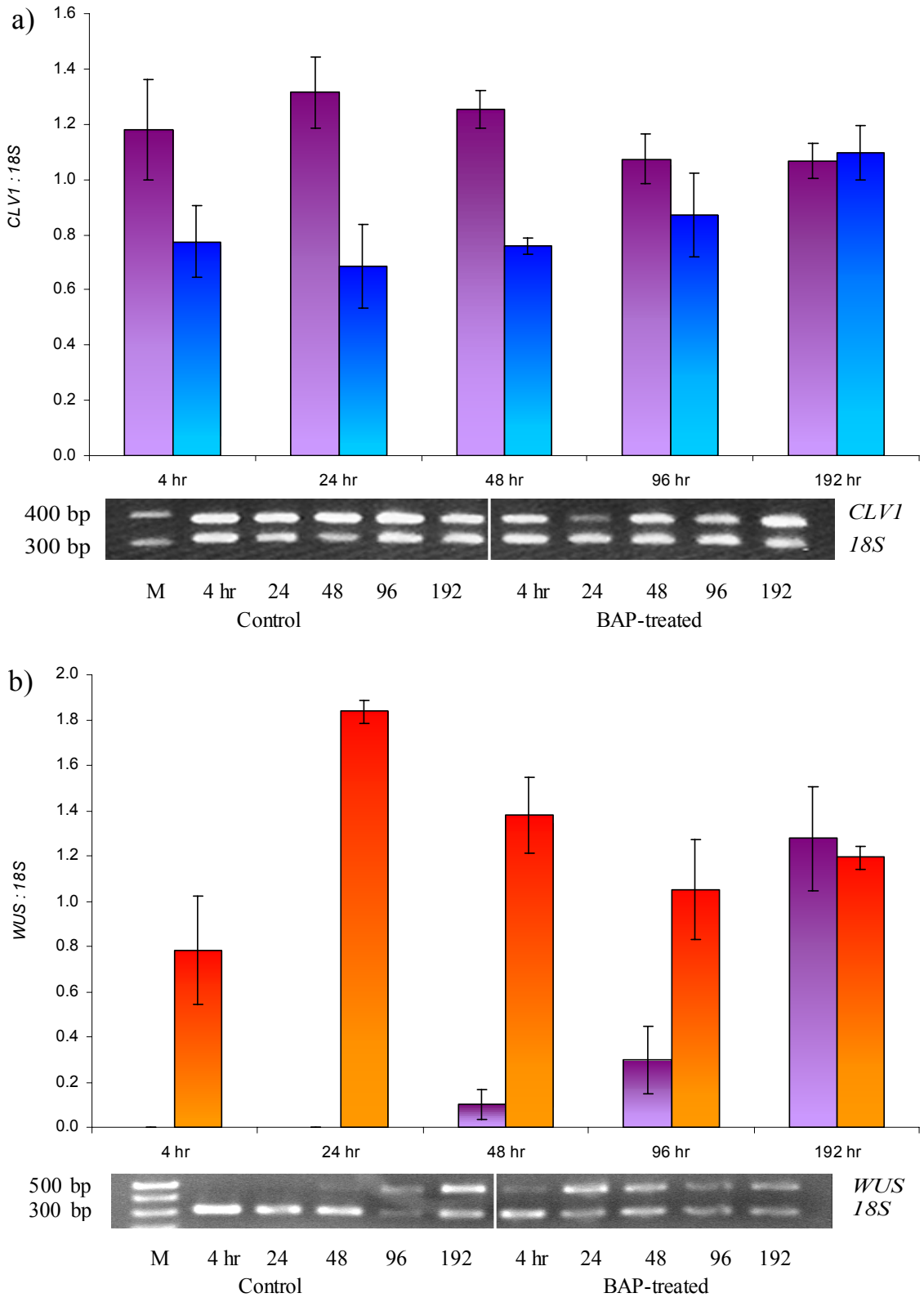


Figure 3.12. RT-PCR amplification of transcript levels of (a) *CLV1* and (b) *WUS* in control (purple) and BAP-treated (blue and red) populations collected at 4, 24, 48, 96, 192 hrs after treatment relative to *18S*, the internal control. M is size marker. N = 3

abundance at 24, 48, and 96 h (p = 0.0007; 0.0019; 0.0482). Transcript abundance of *CLV1* in BAP-treated plants was similar to controls at 96 and 192 hr, as was *WUS* by 192 hr. The BAP-induced changes in *CLV1* and *WUS* transcript abundance coincided with the occurrence of the increased organ number floral phenotype (Figure 3.13a), relative to controls (Figure 3.13b) (Lindsay et al., 2006).

192 h after treatment time, control plants showed increased transcript abundance of *WUS* compared to 96 hours. Gene Chronologer of Genevestigator (Zimmermann et al., 2004) was used to identify typical expression levels of *WUS* (Figure 3.14). Information from the database, representing 22 to 362 arrays, showed consistently low transcript abundance of *WUS* early in *Arabidopsis* development, with a marked increase 25.0-28.9 days after planting, approximately aligning with the 192 h time point (22-26 days after planting) of the RT-PCR time course of this study.

3.3.1.3 *CLV1* Transcript Levels in Specific Tissues

Although primarily investigated for its functions in the regulation of shoot meristem maintenance (Clark et al., 1993; Schoof et al., 2000), RT-PCR determined that *CLV1* is differentially expressed in various organs of wild type plants (Figure 3.15). The lowest expression levels were found in flower buds, coincident with the location and timing of a peak in *WUS* transcript observed in the Gene Chronologer data (Figure 3.14). Highest levels were found in seedlings, at the time equivalent to BAP-treatment.

3.3.1.4 *CLV1* and *WUS* Transcript Levels in *amp1*

The *Arabidopsis* mutant *amp1* has increased endogenous cytokinin levels (Chaudhury et al., 1993). Control, BAP-treated wild type, and *amp1* mutant plants were harvested at the 4-5 leaf stage, as per microarray samples (48 hours after treatment for the BAP-treated samples). Transcript abundance of *CLV1* and *WUS* was measured with RT-PCR. Transcript levels of *CLV1* were decreased in BAP-treated wild type plants, as expected, but remained steady in cytokinin-enriched *amp1*. Transcript levels of *WUS* increased in both BAP-treated plants and *amp1* plants. In summary, there did not appear to be an inverse correlation between *CLV1* and *WUS* expression in *amp1* (Figure 3.16).

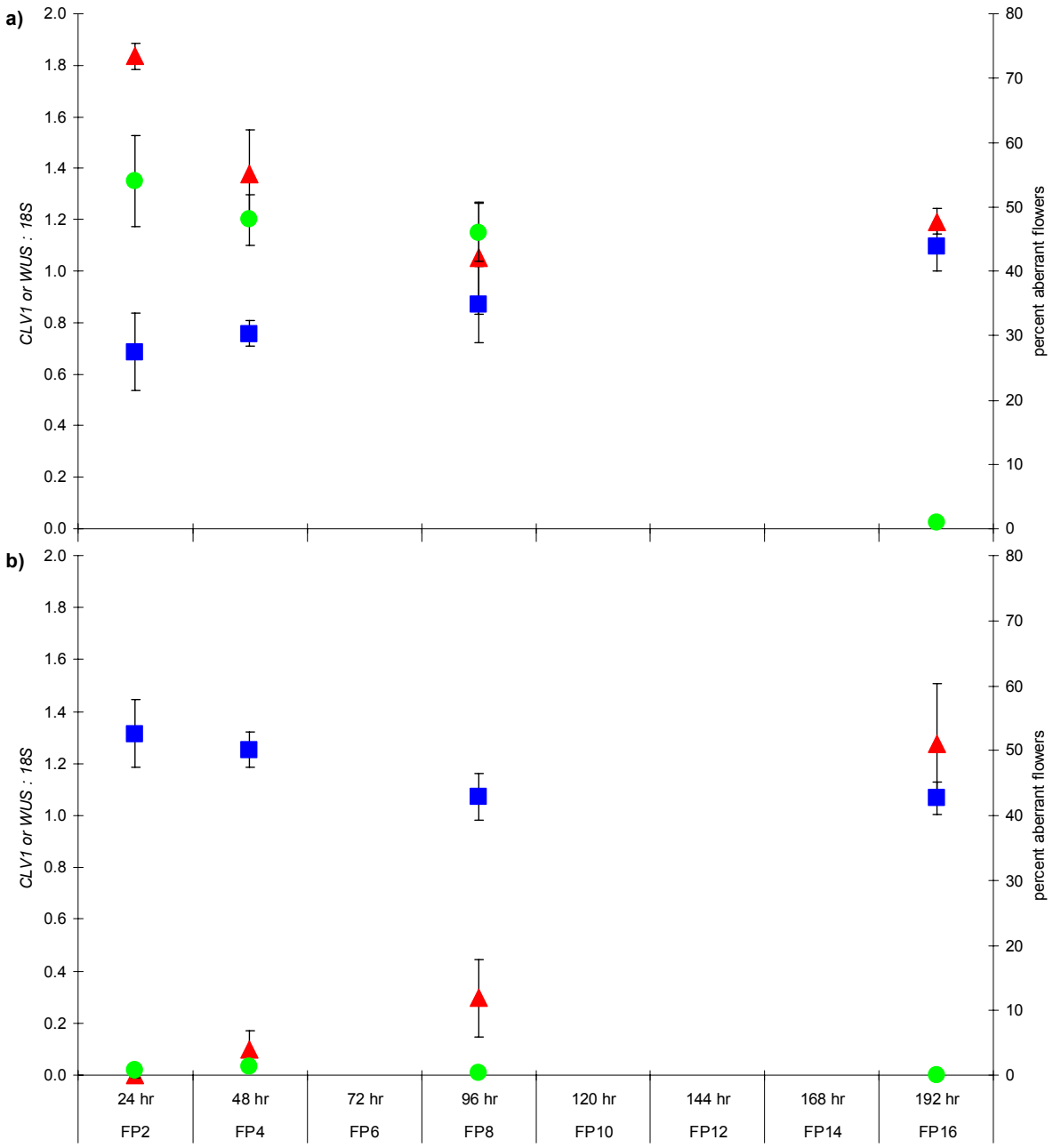
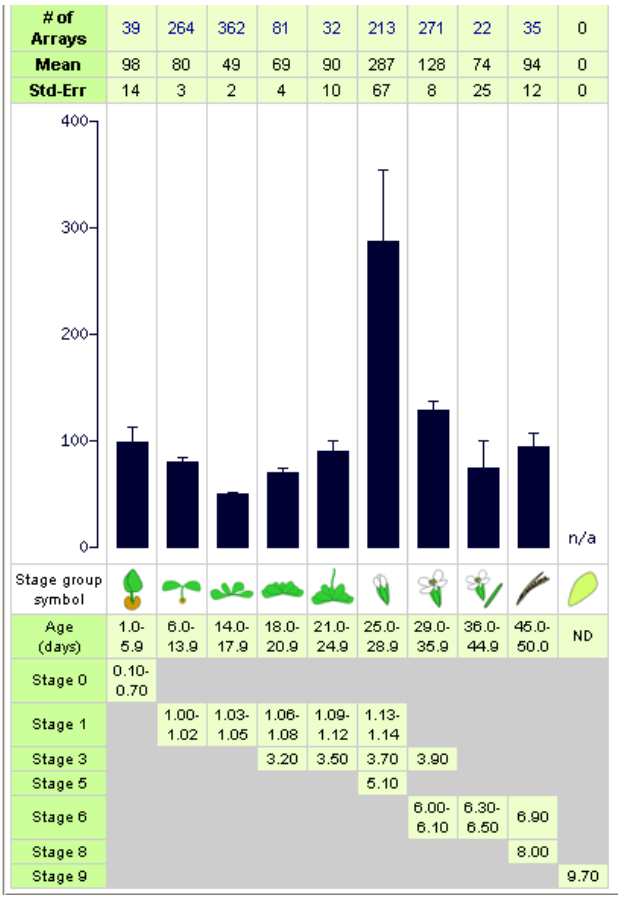


Figure 3.13. Temporal coincidence of BAP-induced changes in transcript levels and floral phenotype. (a) BAP-induced transcript abundance showed decreased *CLV1* (■) and increased *WUS* (▲) coinciding with an increase in the occurrence of aberrant floral organ phenotype (●), relative to (b) control levels. Y-axis scales represent RT-PCR of transcript abundance of genes of interest divided by the *18S* internal control for *CLV1* and *WUS* and percentage of increased floral organ phenotype. X-axis represents a time course from 24 hours after BAP treatment, approximately equivalent to development of flower position 2 (FP2), to 192 hours after treatment, approximately equivalent to FP16.



# of Arrays	39	264	362	81	32	213	271	22	35	0
Mean	98	80	49	69	90	287	128	74	94	0
Std-Err	14	3	2	4	10	67	8	25	12	0
Stage group symbol										
Age (days)	1.0-5.9	6.0-13.9	14.0-17.9	18.0-20.9	21.0-24.9	25.0-28.9	29.0-35.9	36.0-44.9	45.0-50.0	ND
Stage 0	0.10-0.70									
Stage 1		1.00-1.02	1.03-1.05	1.06-1.08	1.09-1.12	1.13-1.14				
Stage 3				3.20	3.50	3.70	3.90			
Stage 5						5.10				
Stage 6							6.00-6.10	6.30-6.50	6.90	
Stage 8									8.00	
Stage 9										9.70

Figure 3.14. Gene Chronologer data of *WUS* (At2g17950) expression through the *Arabidopsis* lifecycle. The increased transcript levels 25.0 to 28.9 days after planting approximately align with the increased levels of *WUS* observed in control samples 192 h after BAP treatment (22-26 days after planting) in the present study.

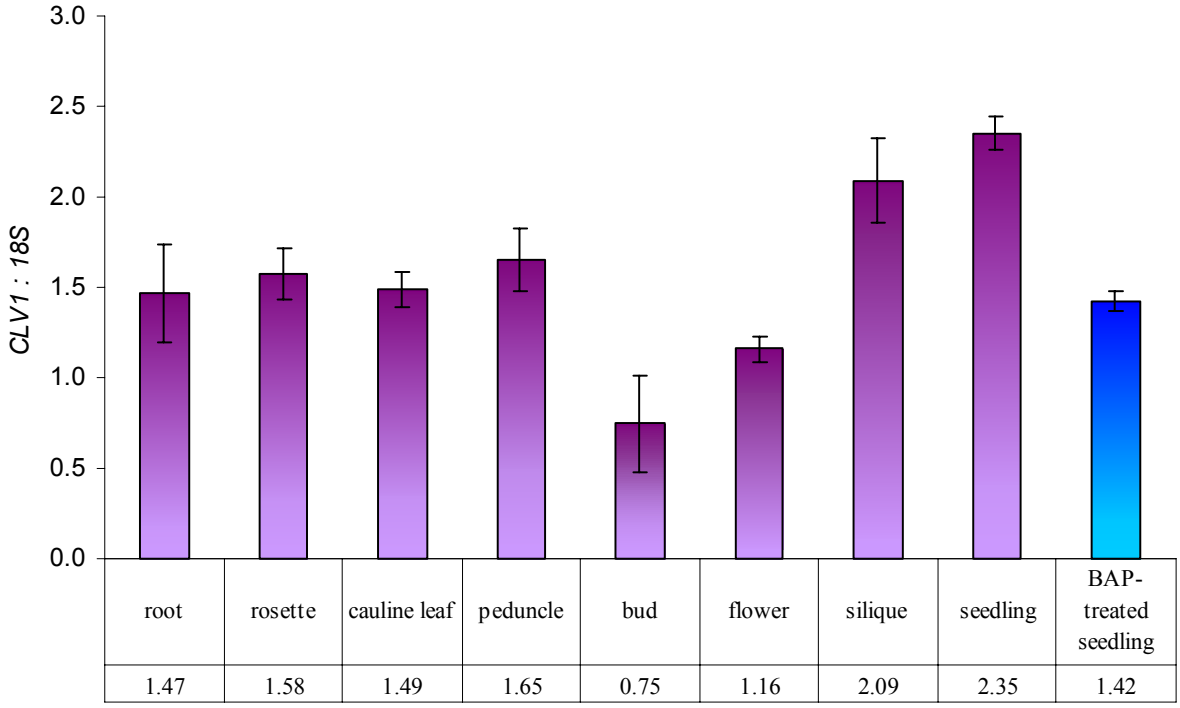
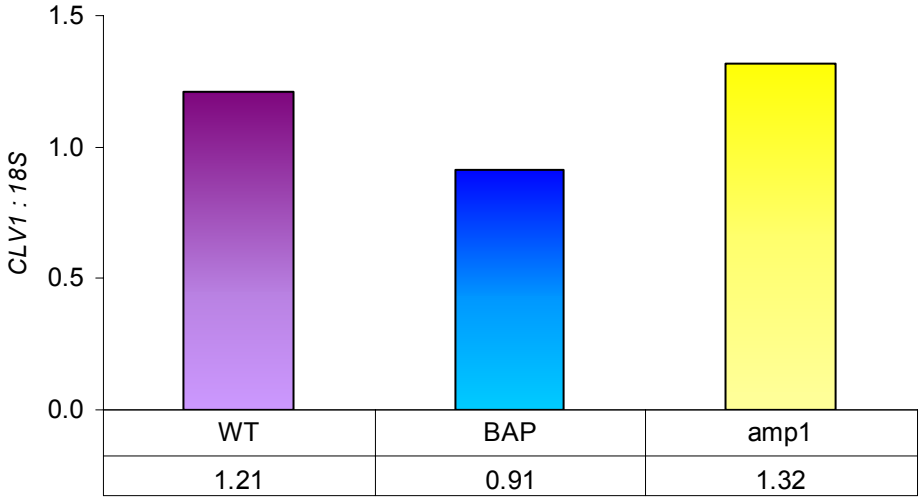


Figure 3.15. Expression of *CLV1* in wild type *Arabidopsis* tissues. Seedling and BAP-treated seedlings were harvested at the equivalent time to samples used in the microarrays (48 h after treatment at 4-5 leaf stage). Y-axis/table represents the mean ratio of the transcript levels of *CLV1* to the *18S* control (N = 3).

a)



b)

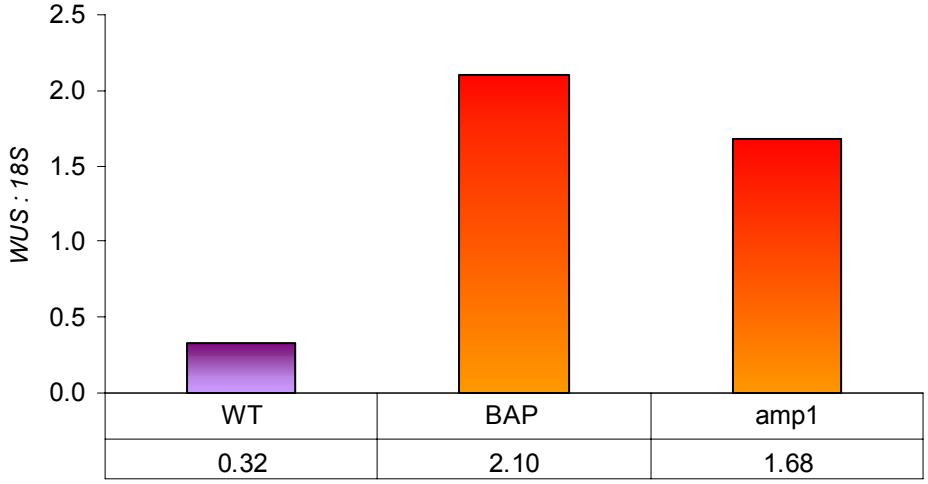


Figure 3.16. Transcript levels of (a) *CLV1* and (b) *WUS* in *Arabidopsis thaliana* Landsberg *erecta* control, BAP-treated wild type, and the cytokinin over-producing mutant *amp1*. Y-axis/table values represent the mean ratio of the transcript levels of *WUS* to the *18S* control (N = 1).

3.3.1.5 *CLV1-LIKE* Transcript Levels

The *Arabidopsis* genome project at TIGR listed *CLV1* as At1g08590 rather than the locus At1g75820 listed in TAIR. Dr. S.E. Clark (personal communication) confirmed *clv1* mutantions concerned At1g75820. The proteins encoded by these two genes share 75% amino acid identity. RT-PCR with primers specific for *CLV1-LIKE* showed that this gene maintained steady state transcript levels throughout the flowering time course in BAP-treated populations (N = 1; Figure 3.17), suggesting independent regulation relative to *CLV1*.

3.3.1.6 *WOX* Subfamily

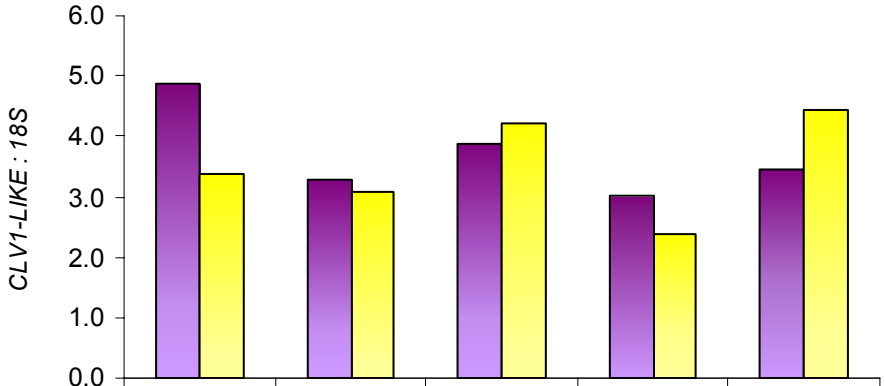
The four members of the *WUSCHEL*-related *WOX* subfamily of homeobox transcription factors listed in TAIR were mined from the microarray data. *WOX5* was not represented on the arrays and *WOX2*, *WOX8*, and *WOX9* were flagged absent.

3.3.1.7 *KNAT* Family

Genes of the *KNAT* family, involved in shoot meristem maintenance and function, were mined from the microarray data. Transcript levels were not affected by the BAP treatment (Table 3.7).

3.3.1.8 Genes Associated with Cytokinesis

While altered transcript levels are the focus of transcriptomic studies, it can also be of interest to note genes and pathways that do not respond to an applied stimulus, in this case BAP. For example, potentially associated with cytokinin regulation of cell division (Riou-Khamlichi et al., 1999), and, thereby, increased apical meristem size, genes associated with cytokinesis were mined from the data. Three *CYCLIN D3* (*CycD3*) genes did not show changes in transcript levels (Table 3.8). *RETINOBLASTOMA-RELATED 1* (*RBR1*), a key component of the cyclin D/retinoblastoma/E2F pathway of the cell cycle (Weinberg, 1995), also maintained control levels. *TSK*, another gene with a role in cell division (Suzuki et al., 2005), was not represented on the arrays.



	4 hr	24 hr	48 hr	96 hr	192 hr
control	4.88	3.27	3.89	3.03	3.44
BAP	3.39	3.08	4.20	2.39	4.44

Figure 3.17. RT-PCR amplification of *CLV1-LIKE* transcript levels in BAP-treated and control populations (N = 1) of *Arabidopsis* over the time course of flowering. Y-axis/table represents the mean ratio of the transcript levels of *CLV1-LIKE* to the *18S* control (N = 1).

Table 3.7. Microarray data of genes of the *KNAT* family

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***SHOOTMERISTEMLESS* (*STM*) At1g62360**												
22k	A	A	A	A	A	311.35	202.65	285.32	237.13	208.14		
								-1.09	-1.31	-1.50	- -	- -
8k	A	A	A	A	A	90.97	93.53	82.13	56.74	17.28		
								-1.11	-1.60	-5.27	- -	
***KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1* (*KNAT1*) At4g08150**												
22k	P	P	P	P	P	879.10	1026.43	547.36	817.75	767.80		
								-1.61	-1.08	-1.14	- -	- -
***KNAT2* At1g70510**												
22k	A	P	P	A	P	88.52	91.43	69.08	110.81	49.61		
								-1.28	1.25	-1.78	- -	- -
8k	8k	A	P	A	A	40.73	37.22	29.07	52.32	27.15		
								-1.40	1.28	-1.50	- -	
***KNAT3* At5g25220**												
22k	P	P	P	P	P	584.54	481.64	379.92	434.36	545.15		
								-1.54	-1.35	-1.07	- -	- -
***KNAT4* At5g11060**												
22k	P	P	P	P	P	989.75	487.30	698.28	797.81	592.34		
								-1.42	-1.24	-1.67	- -	- -
8k	8k	P	P	P	P	981.70	559.29	603.99	733.21	514.23		
								-1.63	-1.34	-1.91	- -	
***KNAT5* At4g32040**												
22k	P	P	P	P	P	642.54	368.54	701.28	896.06	1244.58		
								1.09	1.39	1.94	- -	- -
8k	8k	P	P	P	P	839.13	789.30	1215.24	1584.33	1847.93		
								1.45	1.89	2.20	- -	
***KNAT6* At1g23380**												
22k	A	A	A	A	M	236.56	168.72	212.49	221.61	147.03		
								-1.11	-1.07	-1.61	- -	- -
***KNAT7* At1g62990**												
22k	P	P	P	P	P	390.71	286.53	250.78	216.44	259.56		
								-1.56	-1.81	-1.51	- -	- -

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: -- not recognized as significant.

Table 3.8. Microarray data of genes associated with cytokinesis

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
CYCLIN DELTA-3 (CycD3) **At3g50070**												
22k	P	P	P	P	P	686.80	1057.18	816.28	970.49	771.00		
								1.54	1.19	1.41	- -	- -
CycD3 **At4g34160**												
22k	P	P	P	P	P	1729.96	1652.28	1653.34	1682.04	1532.58		
								-1.05	-1.03	-1.13	- -	- -
8k	P	P	P	P	P	1191.48	1027.91	981.21	689.74	836.75		
								-1.21	-1.73	-1.42	- -	
CycD3 **At5g67260**												
22k	P	P	P	P	P	3095.92	3571.30	3626.53	3779.98	2927.82		
								1.17	1.22	-1.06	- -	- -
RETINOBLASTOMA-RELATED 1 (RBR1) **At3g12280**												
22k	P	P	P	P	P	948.54	927.46	1080.45	953.68	1193.15		
								1.14	1.01	1.26	- -	- -
TONSOKU (TSK) **At3g18730**												
not represented on arrays												

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: -- not recognized as significant.

3.3.2 Floral Meristem Identity and Floral Organ Identity

The microarray data were mined for genes involved in floral development, including floral meristem identity and organ identity genes, as well as those regulating the transcription or spatial influence of these factors (Table 3.9). SAM® and/or GeneSpring® identified that in BAP-treated samples *ANT-LIKE* and *NAP* had significantly increased transcript abundance; *AP1* and *SPL3* had significantly decreased transcript abundance. Numerous genes associated with trichome development mined from the data, including *TRANSPARENT TESTA GLABRA* (*TTG*) and *GLABRA 1* (*GL1*), did not show a significant change in transcript abundance.

3.3.2.1 *AP1* Transcript Levels During Flowering

Floral phenotypes common to *ap1* and BAP-treated wild type plants included ectopic formation of secondary buds in the axils of sepals (Figure 3.10a) and sepals bearing stellate trichomes similar to those found on leaf-like bracts (Figure 3.10g) (see also Venglat and Sawhney 1996). Additionally, in *ap1-1* organs can be under-developed, with a mitten-like appearance, a phenotype occasionally observed in sepals and petals of BAP-treated plants (not shown). RT-PCR of *AP1* over the flowering time course (Figure 3.18) did not show a significant change in *AP1* transcript levels.

3.3.3 Roots

In coordination with other hormones, cytokinins influence root/shoot proliferation. A cursory observation suggested that the roots of BAP-treated plants were shorter and more branched than controls (not shown). A quantitative study may be in order. Genes associated with root function were mined from the microarray data. BAP-treated samples showed significantly increased transcript levels of genes associated with the assimilation, At5g37600, and transport, *AMMONIUM TRANSPORTER 2* (*AMT2*), of NH_4^+ and decreased transcript levels of a gene similar to one associated with nodule-development in *Medicago truncatula*, At1g43650, (Table 3.10). *Arabidopsis RECEPTOR KINASE 3* (*ARK3*), expressed in roots, root-hypocotyl transition zone, axillary buds, and pedicels (Dwyer et al., 1994) and a member of the *SCARECROW* transcription factor family showed significantly higher transcript abundance.

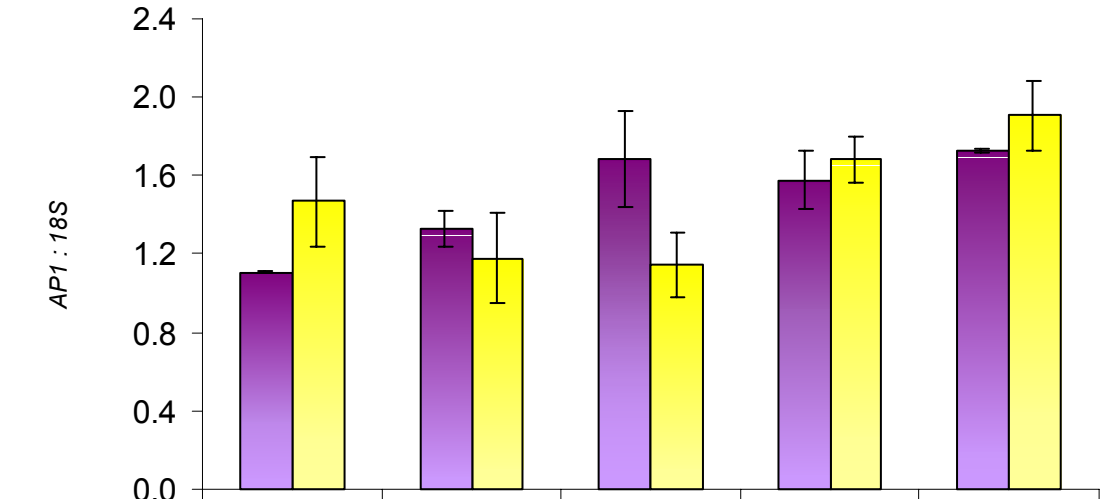
Table 3.9. Microarray data of floral meristem and floral organ identity genes

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***AGAMOUS* (*AG*) At4g18960**												
22k	A	A	A	A	A	45.02	83.89	51.06	64.27	47.80		
								1.13	1.43	1.06	- -	- -
***AINTEGUMENTA* (*ANT*) At4g37750**												
22k	P	P	P	P	P	1290.41	1908.65	1660.10	1585.27	1297.22		
								1.29	1.23	1.01	- -	- -
8k	P	P	P	P	P	343.53	607.01	627.25	560.04	509.29		
								1.83	1.63	1.48	- -	
***AINTEGUMENTA-like* At5g65510**												
22k	A	A	P	P	P	7.63	50.90	81.09	86.43	93.18		
								10.63	11.33	12.21	*	***
***APETALA 1* (*AP1*) At1g69120**												
22k	P	P	P	P	A	314.40	280.88	223.00	214.23	66.56		
								-1.41	-1.47	-4.72	- -	*
8k	P	A	A	A	A	131.71	58.22	53.78	67.79	11.52		
								-2.45	-1.94	-11.43	- -	
***AP2* At4g36920**												
22k	P	P	P	P	P	296.09	373.25	370.91	418.11	444.71		
								1.25	1.41	1.50	- -	- -
8k	P	P	P	P	P	434.50	287.28	268.92	303.60	301.95		
								-1.62	-1.43	-1.44	- -	
***AP3* At3g54340**												
22k	P	A	A	A	A	309.82	224.33	163.68	161.78	121.01		
								-1.89	-1.92	-2.56	- -	- -
***ARGONAUTE* (*AGO1*) At1g48410**												
22k	P	P	P	P	P	3805.61	3515.69	2690.24	3615.25	2504.89		
								-1.41	-1.05	-1.52	- -	- -
8k	P	P	P	P	P	6677.74	6366.91	5432.25	5722.75	4735.83		
								-1.23	-1.17	-1.41	- -	
***CAULIFLOWER* (*CAL*) At1g26310**												
22k	A	A	A	A	A	147.28	90.48	91.60	106.37	72.00		
								-1.61	-1.38	-2.05	- -	- -
8k	A	A	A	A	A	23.08	6.68	21.80	44.21	55.13		
								-1.06	1.92	2.39	- -	
***CURLY LEAF* (*CLF*) At2g23380**												
22k	A	P	P	M	A	255.64	290.30	271.05	221.61	240.81		
								1.06	-1.15	-1.06	- -	- -
8k	A	A	P	M	P	145.29	95.44	93.03	88.43	106.96		
								-1.56	-1.64	-1.36	- -	
***ENHANCER OF AG-4 1* (*HUA1*) At3g12680**												
22k	P	P	P	P	P	711.22	747.44	712.54	560.68	780.51		
								1.00	-1.27	1.10	- -	- -
***HUA2* At5g23150**												
22k	P	P	P	P	P	296.09	257.31	247.78	244.51	255.93		
								-1.19	-1.21	-1.16	- -	- -
***FILAMENTOUS* (*FIL*) At2g45190**												
22k	P	P	P	P	P	998.91	942.55	919.77	916.74	718.19		
								-1.09	-1.09	-1.39	- -	- -

8k	P	P	P	P	P	811.97	505.84	606.89	567.41	502.71		
								-1.34	-1.43	-1.62	- -	
***FRUITFULL* (*FUL*) At5g60910**												
22k	P	P	P	A	A	658.56	681.46	464.77	324.29	502.19		
								-1.42	-2.03	-1.31	- -	- -
8k	P	P	P	P	P	442.65	428.53	436.82	301.39	277.27		
								-1.01	-1.47	-1.60	- -	
***HUA ENHANCER 1* (*HEN1*) At4g20910**												
22k	P	P	P	P	P	331.95	426.97	340.88	319.12	283.77		
								1.03	-1.04	-1.17	- -	- -
***HEN2* At2g06990**												
22k	P	P	P	P	P	486.86	765.35	521.08	614.61	584.47		
								1.07	1.26	1.20	- -	- -
***HEN4* At5g64390**												
22k	P	P	P	P	P	312.11	277.11	249.28	335.37	338.22		
								-1.25	1.07	1.08	- -	- -
***LEAFY* (*LFY*) At5g61850**												
22k	A	P	A	A	A	154.15	150.81	96.11	96.03	12.10		
								-1.60	-1.61	-12.74	- -	- -
8k	A	A	A	A	A	69.25	23.86	25.44	32.42	25.51		
								-2.72	-2.14	-2.72	- -	
***NAC-like ACTIVATED BY AP3/PI* (*NAP*) At1g69490**												
22k	A	A	P	P	P	154.15	87.66	238.01	464.65	870.06		
								1.54	3.01	5.64	*	- -
8k	A	A	P	P	P	153.43	38.18	176.62	251.28	406.45		
								1.15	1.64	2.65	*	
***PERIANTHIA* (*PAN*) At1g68640**												
22k	A	A	A	A	A	62.57	180.03	83.34	141.09	27.23		
								1.33	2.25	-2.30	- -	- -
8k	A	A	P	P	A	99.80	62.99	115.56	103.17	88.86		
								1.16	1.03	-1.12	- -	
***PINOID* (*PID*) At2g34650**												
22k	A	A	P	P	P	223.59	304.44	402.45	634.55	329.75		
								1.80	2.84	1.47	- -	- -
8k	P	P	P	P	P	594.04	583.15	492.06	1176.83	659.86		
								-1.21	1.98	1.11	- -	
***PISTILLATA* (*PI*) At5g20240**												
22k	P	P	P	P	P	191.54	161.18	182.45	197.97	106.49		
								-1.05	1.03	-1.80	- -	- -
8k	A	A	P	P	A	107.27	87.81	77.04	72.22	59.24		
								-1.39	-1.49	-1.81	- -	
***SEPALLATA 1* (*SEP1*) At5g15800**												
22k	A	A	A	A	A	193.83	114.05	111.87	85.69	66.56		
								-1.73	-2.26	-2.91	- -	- -
8k	A	A	A	A	A	114.06	96.40	59.60	88.43	4.11		
								-1.91	-1.29	-27.73	- -	
***SEP2* At3g02310**												
22k	P	A	M	A	A	199.17	126.30	105.87	141.83	74.42		
								-1.88	-1.40	-2.68	- -	- -
8k	A	A	A	A	A	114.74	64.90	13.81	36.11	8.23		
								-8.31	-3.18	-13.95	- -	

SEP3 **At1g24260**												
22k	P	P	P	P	P	270.90	131.96	141.16	138.14	93.78		
								-1.92	-1.96	-2.89	--	--
8k	A	A	A	A	A	75.36	80.17	39.25	75.16	5.76		
								-1.92	-1.00	-13.08	--	
SEP4 **At2g03710**												
22k	A	A	A	A	A	132.78	123.47	42.05	61.31	9.68		
								-3.16	-2.17	-13.72	--	--
8k	P	P	P	P	P	154.79	260.56	71.96	83.27	69.93		
								-2.15	-1.86	-2.21	--	
SEUSS **(*SEU*) At1g43850**												
22k	P	P	P	P	P	931.75	880.34	835.68	1029.76	858.56		
								-1.11	1.11	-1.09	--	--
SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3) **At2g33810**												
22k	P	P	P	P	P	1802.46	1272.44	319.10	452.83	171.83		
								-5.65	-3.98	-10.49	***	**
SPL5 **At3g15270**												
22k	P	P	A	A	A	190.78	123.47	48.80	50.97	87.13		
								-3.91	-3.74	-2.19	--	--
8k	M	P	P	A	P	186.02	93.53	85.76	30.95	34.56		
								-2.17	-6.01	-5.38	--	
SPL9 **At3g57920**												
22k	P	P	A	A	A	193.83	108.39	71.33	59.10	62.92		
								-2.72	-3.28	-3.08	--	--
SUPERMAN **(*SUP*) At3g23130**												
22k	A	A	A	A	A	6.87	59.38	37.54	14.77	35.09		
								5.47	2.15	5.11	--	--
UNUSUAL FLORAL ORGANS **(*UFO*) At1g30950**												
22k	A	A	A	A	A	6.10	5.66	6.01	5.17	6.05		
								-1.02	-1.18	-1.01	--	--
8k	A	A	A	A	A	35.30	35.31	44.34	15.47	34.56		
								1.26	-2.28	-1.02	--	
WIGGUM **(*WIG*) At5g40280**												
22k	A	A	A	P	P	282.35	176.26	359.65	389.30	546.96		
								1.27	1.38	1.94	--	--
8k	P	P	P	P	P	786.18	696.72	875.82	1055.24	1126.36		
								1.11	1.34	1.43	--	
ZWILLE **(*ZLL*) At5g43810**												
22k	P	P	P	P	P	3235.57	2185.76	2180.43	2349.84	1725.59		
								-1.48	-1.38	-1.88	--	--
8k	P	P	P	P	P	1856.81	1541.38	1327.17	1618.96	413.03		
								-1.40	-1.15	-4.50	--	

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.



	4 hr	24 hr	48 hr	96 hr	192 hr
Control	1.11	1.33	1.68	1.58	1.72
BAP-treated	1.47	1.18	1.14	1.68	1.91

Figure 3.18. RT-PCR amplification of *AP1* transcript levels in BAP-treated and control populations of *Arabidopsis* over the flowering time course. Y-axis/table represents the mean ratio of the transcript levels of *AP1* to the 18S control (N = 3).

Table 3.10. Microarray data of genes associated with roots

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
AMMONIUM TRANSPORTER 2 (AMT2) **At2g38290**												
22k	P	P	P	P	P	860.78	528.77	1671.36	2201.36	2086.20		
								1.94	2.56	2.42	***	***
At5g37600 (glutamate-ammonia ligase activity)												
22k	P	P	P	P	P	1143.13	1019.83	3202.31	5108.92	3701.07		
								2.80	4.47	3.24	***	- -
At1g43650 (root nodule development)												
22k	P	P	A	A	A	161.78	169.66	100.61	22.90	12.71		
								-1.61	-7.06	-12.73	*	- -
A. THALIANA RECEPTOR KINASE 3 (ARK3) **At4g21380**												
22k	A	A	P	P	P	96.15	106.51	286.82	364.18	349.72		
								2.98	3.79	3.64	***	***
8k	A	A	P	P	P	16.97	22.91	141.00	150.33	132.46		
								8.31	8.86	7.80	***	
SCARECROW **transcription factor family** **At2g29060**												
22k	P	A	P	P	P	119.81	119.70	289.07	202.41	454.99		
								2.41	1.69	3.80	*	- -
8k	A	M	P	P	M	101.16	69.67	106.84	128.96	319.23		
								1.06	1.27	3.16	- -	

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.

3.3.4 Senescence

Cytokinins delay the developmental processes associated with senescence (Richmond and Lang, 1957; Gan and Amasino, 1995). In this study, BAP-treated plants retained green leaves and siliques approximately 3-10 days longer than controls. As transcriptomic studies have identified over 2000 genes with a 3-fold or greater increase in transcript abundance in relation to senescence (Buchanan-Wollaston et al., 2005), a comprehensive search of senescence-related genes in the BAP-treated data was not undertaken. However, some genes of interest were mined from the data.

Genes associated with the overlapping processes of aging, pathogenesis, and PCD (section 3.3.4), collectively termed SENESCENCE ASSOCIATED GENES (SAGs) (Bleecker and Patterson, 1997), were mined from the data (Table 3.11). Meta Analyzer data indicated that *SENESCENCE ASSOCIATED 1* (*SEN1*), a SAG marker gene for senescent decay (Quirino et al., 2000), has increased transcript levels in senescent tissue and lowered levels in response to zeatin, salicylic acid, and light. Another gene listed by TAIR as related to senescence, At2g21045, had significantly lower transcript abundance in BAP-treated samples.

3.4 BAP and Plant Responses to Environmental Factors

Data analyses by GO indicated that BAP-altered transcript levels emphasized genes associated with plant responses to environmental stimuli. The GO program does not specifically categorize light-responses; however, as might be expected in a study of cytokinin-effects, BAP treatment significantly altered transcript levels of genes associated with light perception and response.

3.4.1 BAP-induced Transcriptome Changes Associated with Light

Components of light-associated pathways, such as photosynthesis, two-component systems, flower timing, and biological clocks were mined individually from the data. Transcript levels of the light receptor proteins PHYA-E and CRY1-2 were not significantly altered by BAP treatment (Table 3.12). *PHOT1* had non-significant lower transcript abundance.

Table 3.11. Microarray data of genes associated with senescence

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***SENESCENCE-ASSOCIATED 1* (*SEN1*) At4g35770**												
22k	P	P	P	P	P	3375.98	1158.39	1729.92	3124.01	880.34		
								-1.95	-1.08	-3.83	- -	- -
8k	P	P	P	P	P	1363.93	394.17	855.47	1292.52	376.00		
								-1.59	-1.06	-3.63	- -	
***SENESCENCE-ASSOCIATED GENE 12* (*SAG12*) At5g45890**												
22k	A	A	A	A	A	15.26	36.76	15.77	33.98	32.07		
								1.03	2.23	2.10	- -	- -
8k	A	A	A	A	A	38.70	3.82	2.91	3.68	10.70		
								-13.31	-10.50	-3.62	- -	
***SAG20* At3g10980**												
22k	P	P	P	P	P	1386.56	854.89	3521.42	3712.76	4476.74		
								2.54	2.68	3.23	***	- -
***SAG21* At4g02380**												
22k	P	P	P	P	P	3421.01	4909.72	5342.94	8613.37	8316.96		
								1.56	2.52	2.43	- -	**
***SAG25 / ELI3-2* At4g37990**												
22k	A	A	P	P	P	246.48	204.53	1489.66	282.93	3112.96		
								6.04	1.15	12.63	*	*
8k	A	A	P	A	P	13.58	33.40	84.31	35.37	153.03		
								6.21	2.60	11.27	- -	
***SAG101* At5g14930**												
22k	P	P	P	P	P	199.93	188.51	307.84	610.18	615.33		
								1.54	3.05	3.08	**	**
***SENESCENCE-RELATED GENE 1* (*SRG1*) At1g17020**												
22k	P	P	P	P	P	137.36	250.72	442.24	296.96	1023.74		
								3.22	2.16	7.45	*	**
At2g21045												
22k	P	P	A	P	P	1085.90	1200.80	257.54	740.93	335.20		
								-4.22	-1.47	-3.24	***	- -

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.

Table 3.12. Microarray data of genes associated with light perception

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***CRYPTOCHROME 1* (*CRY1*) At4g08920**												
22k	P	P	P	P	P	955.41	738.96	843.94	1006.12	1052.18		
								-1.13	1.05	1.10	--	--
8k	P	P	P	P	P	2780.13	1983.28	2538.78	2826.00	2815.50		
								-1.10	1.02	1.01	--	
***CRY2* At1g04400**												
22k	P	P	P	P	P	3519.45	2340.34	2814.13	4309.64	3571.59		
								-1.25	1.22	1.01	--	--
8k	P	P	P	P	P	3351.77	1755.17	2633.99	3344.04	2907.65		
								-1.27	-1.00	-1.15	--	
***PHOTOTROPIN 1* (*PHOT1*) At3g45780**												
22k	P	P	P	P	P	2870.04	1496.76	1371.03	2260.45	757.52		
								-2.09	-1.27	-3.79	--	--
8k	P	P	P	P	P	1678.26	747.31	984.11	1435.48	459.10		
								-1.71	-1.17	-3.66	--	
***PHOT2* At5g58140**												
22k	P	P	P	P	P	1256.84	1153.68	1407.82	829.57	946.29		
								1.12	-1.52	-1.33	--	--
8k	P	P	P	P	P	289.21	371.27	356.14	224.75	263.28		
								1.23	-1.29	-1.10	--	
***PHYTOCHROME A* (*PHYA*) At1g09570**												
22k	P	P	P	P	P	1439.98	1064.13	1164.55	1607.43	1364.98		
								-1.24	1.12	-1.05	--	--
8k	P	P	P	P	P	3108.72	1972.78	2450.83	2481.14	2512.72		
								-1.27	-1.25	-1.24	--	
***PHYB* At2g18790**												
22k	P	P	P	P	P	840.94	991.56	885.23	811.84	1012.85		
								1.05	-1.04	1.20	--	--
8k	P	P	P	P	P	479.31	577.42	477.52	464.25	724.03		
								-1.00	-1.03	1.51	--	
***PHYC* At5g35840**												
22k	P	P	P	P	A	289.98	262.03	379.92	342.02	225.68		
								1.31	1.18	-1.28	--	--
8k	P	P	P	P	P	311.62	229.06	311.08	249.07	402.33		
								-1.00	-1.25	1.29	--	
***PHYD* At4g16250**												
22k	P	P	P	P	P	528.83	433.57	518.08	460.96	739.97		
								-1.02	-1.15	1.40	--	--
8k	P	P	P	P	P	61.78	59.17	56.69	116.43	145.63		
								-1.09	1.88	2.36	--	
***PHYE* At4g18130**												
22k	P	P	P	P	P	1051.56	772.89	1050.42	829.57	1086.06		
								-1.00	-1.27	1.03	--	--
8k	P	P	P	P	M	135.10	97.35	205.69	128.22	227.91		
								1.52	-1.05	1.69	--	

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates.
SAM® and GeneSpring® statistical analysis: -- not recognized as significant.

BAP induced a significant increase in transcript levels of genes functioning in stress responses associated with light, including *DARK INDUCIBLE 2* (*DIN2*), *DIN9*, *RESPONSIVE TO HIGH LIGHT 41* (*RHL41*), *SIGMA FACTOR BINDING PROTEIN 1* (*SIB1*), and *TOUCH 3* (*TCH3*) (Table 3.13). Two members of the *GH3* family, whose expression is induced by auxin and light, showed a BAP-induced increase in transcript abundance. *ELONGATED HYPOCOTYL 5* (*HY5*) showed a non-significant increase in transcript levels. *TT7* was recognized as significant in the BAP-treated replicates.

Other genes associated with light seemed to have been affected by BAP but were not recognized as significant by SAM®. These putative false negatives were interpreted as a consequence of the T_1 generation being used for the C2 sample. These data are presented in the epigenetic inheritance section (section 3.6).

Integral to phytochrome function, three *DE-ETIOLATED* (*DET*) and eight *CONSTITUTIVE PHOTOMORPHOGENIC* (*COP*) genes also did not show significant change in transcript levels in BAP-treated samples. Genes functioning as positive elements in PHYA signalling, *FAR-RED ELONGATED HYPOCOTYL 1* (*FHY1*), *FHY3*, *FAR-RED INSENSITIVE 2* (*FIN2*), *SUPPRESSOR OF PHYA 1* (*SPA1*), *FAR-RED IMPAIRED RESPONSE 1* (*FAR1*), *EMPFINDLICHER IM DUNKELROTEN LICHT 1* (*EID1*), and *PHYTOCHROME A SIGNAL TRANSDUCTION 1* (*PAT1*) (Nagy and Schäfer, 2002), were not significantly altered 48 hours after BAP treatment. *RED LIGHT ELONGATED 1* (*RED1*), *PHYTOCHROME-SIGNALING EARLY FLOWERING 2* (*PEF2*) and *PEF3*, genes affecting PHYB signalling, were also not altered by BAP.

Three proteins that interact directly with phytochromes, known collectively as phytochrome signalling partners (Nagy and Schäfer, 2002), *PHYTOCHROME INTERACTING FACTOR 3* (*PIF3*), *PHYTOCHROME KINASE SUBSTRATE 1* (*PSK1*), and *NUCLEOSIDE DIPHOSPHATE KINASE 2* (*NDPK2*) also maintained steady state transcript levels in the BAP-treated samples.

3.4.2 BAP-induced Transcript Changes Associated with Defense

GO indicated that the transcriptomic response to BAP treatment emphasised genes functioning in defense and stress (Table 3.14). Meta Analyzer data indicated a correlation between stress-response genes affected by BAP and responses to biotic

Table 3.13. Microarray data of genes associated with light response

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
GH3-12 **At5g13320**												
22k	A	A	P	P	P	6.10	3.77	1488.91	1470.03	3112.96		
								243.89	240.80	509.92	**	***
GH3-14 **At5g13360**												
22k	P	P	P	P	P	201.46	267.68	695.27	378.22	1433.96		
								3.45	1.88	7.12	*	**
DARK INDUCIBLE 2 **(*DIN2*) At3g60140**												
22k	P	P	P	P	P	71.73	58.44	906.26	630.86	3616.36		
								12.63	8.79	50.41	*	**
8k	A	A	A	A	P	109.98	10.50	154.09	120.85	553.72		
								1.40	1.10	5.03	--	
DIN9 **At1g67070**												
22k	P	P	P	P	P	186.20	272.40	749.33	466.87	1366.19		
								4.02	2.51	7.34	*	**
ELONGATED HYPOCOTYL 5 **(*HY5*) At5g11260**												
22k	P	P	P	P	P	137.36	214.90	304.84	141.09	297.68		
								2.22	1.03	2.17	--	--
8k	A	A	P	A	P	15.61	42.95	103.21	16.21	106.96		
								6.61	1.04	6.85	--	
RESPONSIVE TO HIGH LIGHT 41 **(*RHL41/ZAT12*) At5g59820**												
22k	P	P	P	P	P	232.75	195.11	2425.20	815.54	4526.96		
								10.42	3.50	19.45	*	**
8k	A	P	P	P	P	143.93	84.94	1455.82	478.98	3456.43		
								10.11	3.33	24.01	*	
SIGMA FACTOR BINDING PROTEIN 1 **(*SIB1*) At3g56710**												
22k	P	P	P	P	P	660.09	606.06	3221.83	3511.09	2907.85		
								4.88	5.32	4.41	***	***
TOUCH 3 **(*TCH3*) At2g41100**												
22k	P	P	P	P	P	3924.66	2738.09	12119.98	16360.23	15302.22		
								3.09	4.17	3.90	***	***
8k	P	P	P	P	P	12901.97	11201.98	28157.71	29568.03	29558.62		
								2.18	2.29	2.29	--	
TRANSPARENT TESTA 7 **(*TT7*) At5g07990**												
22k	A	P	P	M	P	147.28	238.46	963.32	173.60	831.94		
								6.54	1.18	5.65	*	--

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

Table 3.14. Microarray data of genes associated with abiotic & biotic stimuli and stress response

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal	R2Signal	R3Signal	SAM	Gene-Spring
								R1:C1	R2:C1	R3:C1		
ACTIVATED DISEASE RESISTANCE 1 (ADR1) **At1g33560**												
22k	A	A	P	P	P	210.62	204.53	806.40	540.00	902.12		
								3.83	2.56	4.28	***	***
ALTERNATIVE OXIDASE 1a (AOX1a) **At3g22370**												
22k	P	P	P	P	P	531.89	425.09	3915.61	2760.56	10950.13		
								7.36	5.19	20.59	*	**
8k	P	P	P	P	P	393.77	329.27	3411.69	2133.32	10607.07		
								8.66	5.42	26.94	*	
AVRRPT2-INDUCED GENE 1 (AIG1) **At1g33960**												
22k	A	A	P	P	P	19.08	68.81	3055.90	3361.13	5887.10		
								160.18	176.18	308.59	***	***
8k	A	P	P	P	P	135.10	106.89	150.45	151.06	225.44		
								1.11	1.12	1.67	- -	
BLUE-COPPER-BINDING PROTEIN (BCB) **At5g20230**												
22k	P	P	P	P	P	1182.81	2040.61	7693.06	4647.23	11356.11		
								6.50	3.93	9.60	**	**
8k	P	P	P	P	P	1114.09	1954.64	7835.84	5070.60	13470.29		
								7.03	4.55	12.09	**	
BON ASSOCIATION PROTEIN 1 (BAP1) **At3g61190**												
22k	P	P	P	P	P	213.67	131.01	490.30	477.95	850.09		
								2.29	2.24	3.98	**	**
BONZAI 1 (BON1) **At5g61900**												
22k	P	P	P	P	P	337.29	351.57	727.56	1133.18	1142.93		
								2.16	3.36	3.39	***	- -
CYSTEINE-RICH RLK 11 (CRK11) **At4g23190**												
22k	P	P	P	P	P	79.36	109.34	175.70	221.61	435.03		
								2.21	2.79	5.48	*	**
ECS1 **At1g31580**												
22k	P	P	P	P	P	573.86	914.27	2296.80	2074.30	2022.06		
								4.00	3.61	3.52	***	- -
8k	P	P	P	P	P	2012.28	1657.82	6834.28	6369.75	6064.59		
								3.40	3.17	3.01	***	
ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) **At3g48090**												
22k	M	P	P	P	P	268.61	254.49	1074.45	2562.59	1731.04		
								4.00	9.54	6.44	**	***
8k	A	P	P	P	P	118.13	135.53	403.38	1075.87	668.91		
								3.41	9.11	5.66	**	
GDSL-motif LIPASE/HYDROLASE PROTEIN 1 (GLIP1) **At5g40990**												
22k	A	A	P	P	P	35.10	58.44	293.58	154.39	458.63		
								8.36	4.40	13.07	*	**
GLUTATHIONE S-TRANSFERASE 6 (GSTF6) **At1g02930**												
22k	P	P	P	P	P	2280.92	4152.85	17509.48	9420.78	17370.88		
								7.68	4.13	7.62	***	**

GSTZ1 **At2g02390**

22k	P	P	P	P	P	382.32	319.52	1237.38	710.64	1940.38		
								3.24	1.86	5.08	*	**
8k	P	P	P	P	P	486.10	589.83	1207.25	914.49	2047.86		
								2.48	1.88	4.21	- -	

HEAT SHOCK FACTOR 4 (HSF4) **At4g36990**

22k	P	P	P	P	P	257.93	422.26	2395.92	1441.96	3660.53		
								9.29	5.59	14.19	**	**

HEAT SHOCK PROTEIN 81-1 (HSP81-1) **At5g52640**

22k	P	P	P	P	P	177.80	150.81	1407.82	399.64	3832.97		
								7.92	2.25	21.56	*	**
8k	P	P	P	P	P	244.41	141.25	2740.11	618.99	6335.28		
								11.21	2.53	25.92	*	

NITRILASE 4 (NIT4) **At5g22300**

22k	A	A	P	P	P	248.77	216.79	1228.37	828.83	1538.03		
								4.94	3.33	6.18	***	**
8k	P	P	P	P	P	190.77	112.62	853.29	470.88	1196.30		
								4.47	2.47	6.27	**	

PATATIN-like PROTEIN 2 (PLP2) **At2g26560**

22k	P	P	P	P	P	393.76	262.03	1253.89	1925.08	2025.69		
								3.18	4.89	5.14	***	**

PATHOGENESIS-RELATED 1 (PR1) **At2g14610**

22k	P	P	P	P	P	171.70	399.64	5062.13	7477.23	3618.18		
								29.48	43.55	21.07	***	***

PR2 **At3g57260**

22k	P	P	P	P	P	182.38	310.10	4591.36	13444.54	4814.35		
								25.17	73.72	26.40	*	***
8k	A	P	P	P	P	174.48	154.62	5394.45	17709.87	6061.30		
								30.92	101.50	34.74	*	

PR5 **At1g75040**

22k	M	P	P	P	P	279.30	261.08	2983.07	4969.31	3807.56		
								10.68	17.79	13.63	***	***

Pathogenesis-related protein 1 (PR-1) **precursor At4g33720**

22k	P	P	P	P	A	3786.53	1052.82	73.58	503.80	11.50		
								-51.46	-7.52	-329.38	**	**
8k	P	P	A	P	M	1518.72	444.76	42.88	305.08	41.96		
								-35.42	-4.98	-36.19	*	

PBS3 **(for avrPphB susceptible) At5g13320**

22k	A	A	P	P	P	6.10	3.77	1488.91	1470.03	3112.96		
								243.89	240.80	509.92	**	***

PHYTOALEXIN DEFICIENT 3 (PAD3) **At3g26830**

22k	A	P	P	P	P	176.28	301.61	2808.87	1355.53	4271.63		
								15.93	7.69	24.23	**	***

PAD4 **At3g52430**

22k	A	A	P	P	P	61.81	77.29	762.85	1400.60	955.37		
								12.34	22.66	15.46	***	***

RECEPTOR-LIKE PROTEIN KINASE 1 (RLK1) **At5g60900**

22k	M	P	P	P	P	122.86	87.66	487.29	919.69	499.16		
								3.97	7.49	4.06	**	***
8k	A	A	P	P	P	124.92	32.45	279.83	364.03	297.84		
								2.24	2.91	2.38	***	

RLK5 **At4g23140**												
22k	P	P	P	P	P	363.24	345.91	640.46	872.42	928.75		
								1.76	2.40	2.56	***	***
SALICYLIC ACID IINDUCTION DEFICIENT 1 **(*SID1*) At4g39030**												
22k	P	P	P	P	P	250.30	280.88	1094.72	1577.89	1679.61		
								4.37	6.30	6.71	***	***
8k	P	P	P	P	P	35.30	92.58	359.78	614.57	703.46		
								10.19	17.41	19.93	***	
SID2 **(=*ICS1*) At1g74710**												
22k	P	P	P	P	P	281.59	246.00	2026.50	2993.99	3209.16		
								7.20	10.63	11.40	***	***
SULFURTRANSFERASE **(*ST*) At2g03760**												
22k	P	P	P	P	P	560.88	426.97	1043.66	1978.27	2285.86		
								1.86	3.53	4.08	**	**
8k	P	P	P	P	P	358.46	172.75	656.32	1198.93	1212.75		
								1.83	3.34	3.38	**	
SYNAPTOSOMAL-ASSOCIATED PROTEIN 33 **(*SNAPP33*) At5g61210**												
22k	P	P	P	P	P	1527.74	1341.24	3103.20	3272.49	6078.90		
								2.03	2.14	3.98	**	**
8k	P	P	P	P	P	2060.49	1319.96	3070.09	2779.58	4234.77		
								1.49	1.35	2.06	*	
TOLL/INTERLEUKIN-1 RECEPTOR-LIKE **(*TIR*) At1g72930**												
22k	P	P	P	P	P	1203.42	1933.16	4303.04	4603.65	3720.43		
								3.58	3.83	3.09	***	***
8k	P	P	P	P	P	936.21	1117.62	2511.89	2893.80	2504.49		
								2.68	3.09	2.68	***	
TRYPSIN INHIBITOR 1 **(*TTI1*) At2g43510**												
22k	P	P	P	P	P	464.73	583.44	2555.09	781.56	3195.25		
								5.50	1.68	6.88	*	**
8k	P	P	P	P	P	544.48	434.26	3136.95	759.00	3246.63		
								5.76	1.39	5.96	*	
TYROSINE AMINOTRANSFERASE 3 **(*TAT3*) At2g24850**												
22k	A	P	P	P	P	106.83	168.72	1200.59	709.90	1559.20		
								11.24	6.64	14.59	***	**
WOUND-INDUCED PROTEIN 12 **(*SAG20*) At3g10980**												
22k	P	P	P	P	P	1386.56	854.89	3521.42	3712.76	4476.74		
								2.54	2.68	3.23	***	--
YELLOW-LEAF-SPECIFIC GENE 9 **(*YLS9*) At2g35980**												
22k	A	A	P	P	P	22.13	14.14	882.23	265.20	2152.75		
								39.87	11.98	97.28	*	**
8k	A	A	P	P	P	410.74	147.93	1213.06	372.87	3186.56		
								2.95	-1.10	7.76	*	

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

elicitors and ozone (Figure 3.19). Genes associated with disease resistance were also mined from the significant data (Table 3.15). Meta Analyzer data indicated that two of the genes in Tables 3.14 and 3.15 respond to zeatin-treatment (Yoshida data), *YELLOW-LEAF-SPECIFIC GENE 9* (*YLS9*) and disease resistance At1g57650. Meta Analyzer data also showed that ethylene increases transcript abundance of *BLUE-COPPER BINDING PROTEIN* (*BCB*), *CYSTEINE-RICH RLK 11* (*CRK11*), and *PAD3*; salicylic acid increases transcripts *AVRRPT2-INDUCED GENE 1* (*AIG1*), *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *GST18*, *PHYTOALEXIN DEFICIENT 3* (*PAD3*), *SULFURTRANSFERASE* (*ST*), *TOLL/INTERLEUKIN-1 RECEPTOR-LIKE* (*TIR*) and *PATHOGENESIS- RELATED 1* (*PR1*).

While most defense response genes showed increased transcript levels, a gene listed by TAIR as a precursor to *PR1* production was an exception, with BAP inducing lowered transcript levels. Meta Analyzer indicated that this gene also had lowered transcript levels in plants treated with salicylic acid (data not shown), a hormone that induces *PR1* (Malamy et al., 1990). *AIG1*, a gene with one of the greatest increases in transcript levels in the 22k data, is associated with pathogen-specific response to attack by *Pseudomonas syringae* van Hall (Reuber and Ausubel, 1996). In a rare case of the technical replicates conflicting, the 8k data indicated maintenance of steady state transcript levels (Table 3.14).

3.4.2.1 Cell Wall

GO identified an emphasis on genes associated with cell wall function. A Caffeoyl-CoA O-methyltransferase involved in lignin biosynthesis and repressed by chilling (Zhong et al., 2000; Provart et al., 2003) had significantly increased levels in BAP-treated samples (Table 3.16). *ESC1*, which has increased expression in response to the pathogen *Xanthomonas campestris* L. (Aufsatz and Grimm, 1994) also showed increased levels in BAP-treated samples. However, transcript levels of *LIPOXYGENASE 1* (*LOX1*), *LOX2* and *LOX3*, associated with cell wall function as part of a defense response, were not significantly altered by BAP.

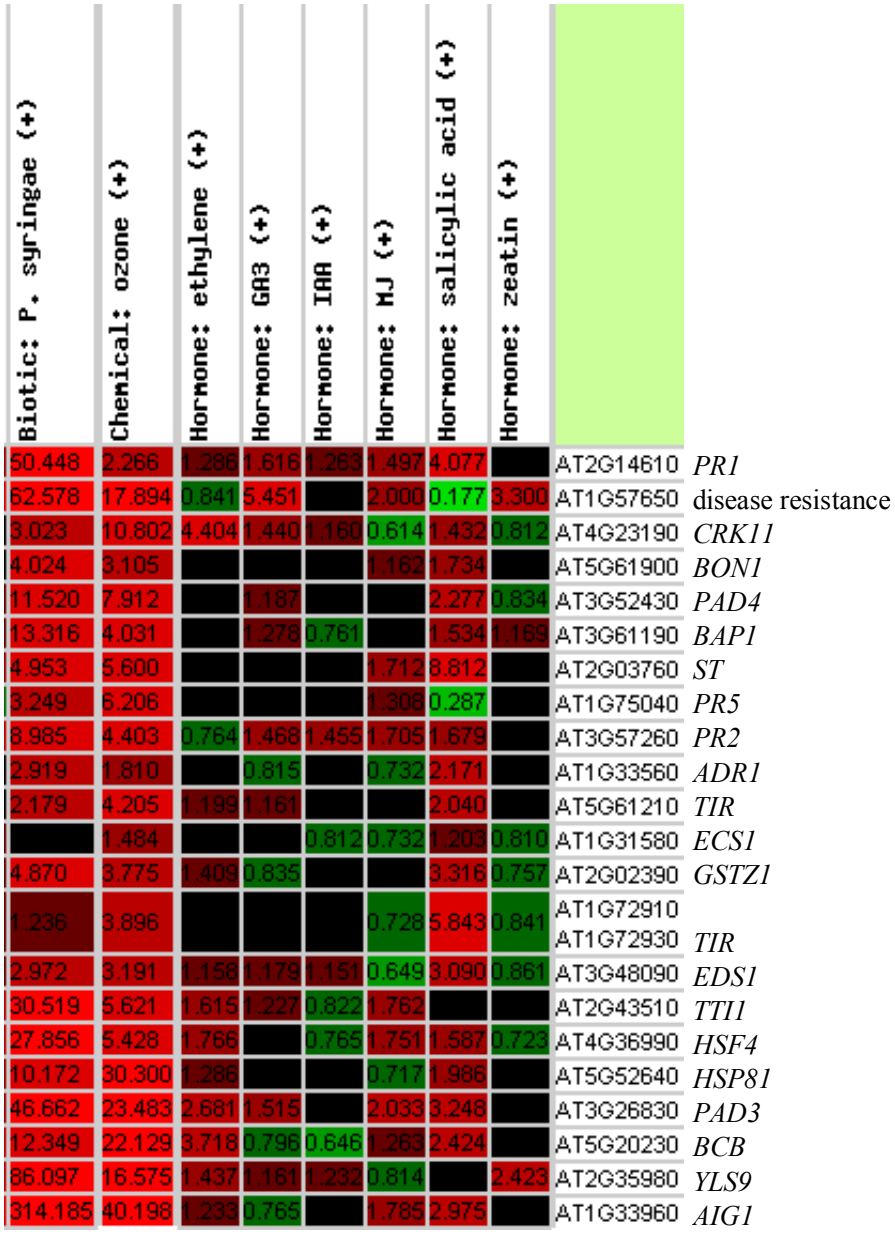


Figure 3.19. Meta Analyzer data of changes in transcript abundance in response to various hormones, *Pseudomonas syringae*, and ozone. These defense-response genes showed increased transcript abundance in BAP-treated tissues of the present study.

Table 3.15. Microarray data of genes associated with disease resistance

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
disease resistance protein At1g56510												
22k	A	P	P	P	P	157.96	143.27	424.22	464.65	572.37		
								2.69	2.94	3.62	***	***
disease resistance protein At1g57650												
22k	A	A	P	P	P	13.74	12.25	1202.09	1102.16	2301.60		
								87.51	80.24	167.56	**	***
disease resistance protein At2g32680												
22k	A	A	P	P	P	67.15	45.24	764.35	2689.65	1035.84		
								11.38	40.05	15.42	*	**
8k	A	A	P	P	P	33.95	18.13	1552.49	4388.96	1822.42		
								45.73	129.29	53.69	**	
disease resistance protein At3g05650												
22k	P	P	P	P	P	153.38	126.30	410.71	485.33	491.90		
								2.68	3.16	3.21	***	--
disease resistance protein At3g11010												
22k	A	A	P	P	P	63.34	50.90	397.94	900.49	433.82		
								6.28	14.22	6.85	**	***
disease resistance protein At3g23110												
22k	A	A	P	P	P	5.34	20.74	157.68	537.04	484.04		
								29.52	100.54	90.61	**	***
disease resistance protein At3g25010												
22k	P	P	P	P	P	85.47	94.25	226.00	498.63	262.59		
								2.64	5.83	3.07	*	**
disease resistance protein At4g13900												
22k	A	A	P	P	P	8.39	39.59	115.63	192.80	249.88		
								13.77	22.97	29.77	**	--
8k	A	A	A	A	A	27.16	25.77	61.78	42.00	84.74		
								2.27	1.55	3.12	*	
disease resistance protein At5g40170												
22k	P	P	P	P	P	289.98	291.25	617.94	723.20	1052.78		
								2.13	2.49	3.63	**	--
disease resistance protein At1g57650												
22k	A	A	P	P	P	13.74	12.25	1202.09	1102.16	2301.60		
								87.51	80.24	167.56	**	***

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

Table 3.16. Microarray data of genes associated with the cell wall

	C1	C2	R1	R2	R3	C1Signal	C2 Signal	R1Signal R1/C1Fold	R2Signal R2/C1Fold	R3Signal R3/C1Fold	SAM	GeneSpring
caffeoyl-CoA O-methyltransferase At1g67980												
22k	P	P	P	P	P	92.34	155.52	522.58	462.43	878.53		
								5.66	5.01	9.51	**	**
CELL WALL-ASSOCIATED KINASE 1 (WAK1) At1g21250												
22k	P	P	P	P	P	476.18	918.98	5178.51	6971.95	5553.72		
								10.88	14.64	11.66	***	***
8k	P	P	P	P	P	1236.97	2562.61	10661.71	12684.96	11948.18		
								8.62	10.25	9.66	***	
***ECS1* At1g31580**												
22k	P	P	P	P	P	573.86	914.27	2296.80	2074.30	2022.06		
								4.00	3.61	3.52	***	- -
8k	P	P	P	P	P	2012.28	1657.82	6834.28	6369.75	6064.59		
								3.40	3.17	3.01	***	
At2g45220												
22k	A	M	P	P	P	60.29	305.38	1863.57	610.18	4287.36		
								30.91	10.12	71.12	*	**
8k	P	P	P	P	P	179.91	191.84	1244.31	381.71	2525.89		
								6.92	2.12	14.04	*	
At3g14060												
22k	M	P	P	P	P	199.93	188.51	464.77	817.75	1056.41		
								2.32	4.09	5.28	**	**

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

3.4.2.2 Detoxification

BAP significantly increased transcript levels of genes encoding cytochrome P450s (Table 3.17), which serve in the early stages of detoxification processes (Marrs, 1996). The *GST* family encodes multifunctional dimeric enzymes associated with detoxification of endogenous and xenobiotic compounds (Wagner et al., 2002). TAIR lists 51 genes in *Arabidopsis* encoding GST protein activity. Twelve of these were not present on the arrays or were flagged for elimination from analysis by SAM®. BAP treatment significantly increased the transcript levels of 13 *GST* genes and decreased the levels of one (Table 3.17 – naming convention according to Wagner et al. (2002)). *GSTF12* appeared to have increased transcript abundance after BAP treatment that was masked by potential epigenetic inheritance affecting transcript levels in the second control. *GSTU5*, which showed a slight, but not significant, increase in transcript levels after BAP treatment, has been shown to be induced by auxin (Kop et al., 1996).

BAP treatment also significantly increased the transcript abundance of *MULTI-DRUG RESISTANCE PROTEIN 7* (*MRP7*) (Table 3.17), encoding an ATP-energized ABC transporter pump active in moving glutathione-S conjugates across the vacuolar membrane (Liu et al., 2001).

3.5 Regulation of Signal Transduction

The evolution of complex multicellular organisms relied on the development of intricate regulatory systems featuring receptors interacting with environmental and intercellular cues, and subsequent growth responses regulated by several factors including hormones. In *Arabidopsis*, 20% of the genome is thought to encode proteins dedicated to signal transduction or transcription regulation (Bevan and Walsh, 2005). Families of transcription factors unique to plants are thought to play important roles in development and physiology (Eulgem et al., 2000) and hormones regulating these signal transduction pathways have a complex evolutionary history (Gazzarrini and McCourt, 2003). Much remains to be determined about the mode of cytokinin action; therefore, the microarray data was mined for BAP-targeted regulation elements in relation to hormone crosstalk and signal transduction, especially receptor kinases, and transcription factors.

Table 3.17. Microarray data of genes associated with cellular detoxification

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
Cytochrome P450 At3g19270												
22k	A	A	M	P	P	7.63	106.51	292.83	214.23	364.84		
								38.37	28.07	47.81	**	***
Cytochrome P450 At2g30750												
22k	A	A	P	P	P	41.97	73.52	1190.82	718.03	2397.80		
								28.37	17.11	57.13	**	***
Cytochrome P450 At2g30770												
22k	A	P	P	P	P	16.79	108.39	1582.01	690.69	2222.94		
								94.23	41.14	132.41	**	***
8k	A	A	P	P	P	36.66	66.81	1428.20	602.78	1954.89		
								38.96	16.44	53.32	**	
Cytochrome P450 At2g34500												
22k	A	A	P	P	P	163.30	208.30	2095.58	590.97	4540.27		
								12.83	3.62	27.80	*	**
GSTF2 **AT4g02520**												
8k	P	P	P	P	P	1976.98	2582.65	8649.88	5432.41	16243.01		
								4.38	2.75	8.22	*	**
GSTF3 **At2g02930**												
22k	P	P	P	P	P	4693.10	7010.65	14398.77	10419.52	19244.70		
								3.07	2.22	4.10	**	**
GSTF6 **At1g02930**												
22k	P	P	P	P	P	2280.92	4152.85	17509.48	9420.78	17370.88		
								7.68	4.13	7.62	***	**
GSTF7 **At1g02920**												
8k	P	P	P	P	P	608.30	781.67	5257.08	1944.67	8618.45		
								8.64	3.20	14.17	*	*
GSTF12 **At5g17220**												
22k	P	P	P	P	P	195.36	641.87	1614.30	202.41	1037.05		
								8.26	1.04	5.31	- -	- -
GSTF14 **At1g49860**												
22k	P	P	P	P	P	1126.35	1003.81	107.37	375.27	340.04		
								-10.49	-3.00	-3.31	***	- -
GSTU1 **At2g29490**												
22k	P	P	P	P	P	193.07	197.93	752.34	358.27	1872.01		
								3.90	1.86	9.70	*	*
8k	A	P	P	P	P	93.69	179.43	400.48	205.59	1144.46		
								4.27	2.19	12.22	*	
GSTU3 **At2g29470**												
22k	A	A	P	P	P	26.71	12.25	1942.41	467.60	5621.48		
								72.73	17.51	210.47	*	**
8k	A	A	P	P	P	17.65	10.50	554.56	178.33	1935.96		
								31.42	10.10	109.68	*	
GSTU4 **At2g29460**												
22k	A	A	P	P	P	6.10	28.28	226.00	124.10	504.61		
								37.02	20.33	82.66	*	**
8k	A	A	P	P	P	44.13	37.22	447.72	147.38	786.56		
								10.15	3.34	17.82	*	

GSTU5 **At2g29450**												
22k	P	P	P	P	P	713.50	994.39	1892.10	525.22	2329.43		
								2.65	-1.36	3.26	- -	- -
8k	P	P	P	P	P	754.27	544.02	1920.26	427.40	2011.66		
								2.55	-1.76	2.67	- -	
GSTU8 **At3g09270**												
22k	P	P	P	P	P	432.68	1287.52	2730.04	1566.81	7004.62		
								6.31	3.62	16.19	*	**
GSTU10 **At1g74590**												
22k	A	A	P	P	P	73.26	89.54	1076.70	728.37	2024.48		
								14.70	9.94	27.63	**	**
GSTU11 **At1g69930**												
22k	A	P	P	P	P	95.39	179.08	1527.20	1528.39	2386.91		
								16.01	16.02	25.02	***	***
GSTU24 **At1g17170**												
22k	P	P	P	P	P	207.56	363.82	2302.81	661.88	6702.70		
								11.09	3.19	32.29	*	**
GSTU25 **At1g17180**												
22k	A	P	P	P	P	6.87	127.24	711.04	205.36	2772.32		
								103.53	29.90	403.66	*	**
GSTZ1 **At2g02390**												
22k	P	P	P	P	P	382.32	319.52	1237.38	710.64	1940.38		
								3.24	1.86	5.08	*	**
8k	P	P	P	P	P	486.10	589.83	1207.25	914.49	2047.86		
								2.48	1.88	4.21	- -	
MRP7 **At3g13100**												
22k	A	A	P	P	P	60.29	69.75	413.71	780.08	845.25		
								6.86	12.94	14.02	***	**

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.

3.5.1 Hormone Crosstalk

In this study, whole axes, including roots, a primary site of cytokinin biosynthesis, were harvested for transcriptomic analyses. Nine genes encoding *IPT* enzymes catalyzing cytokinin biosynthesis (Kakimoto, 2001) were flagged A(bsent) and/or did not have significant changes in transcript levels. Of the seven *CKXs*, which catalyze the removal of the N^6 side chain from cytokinins, and, thereby, function in the degradation of abundant cytokinins (Schmülling et al., 2003) listed in TAIR, three had significantly increased transcript levels, *CKX3*, *CKX4*, and *CKX5*, in BAP-treated plants (Table 3.18).

Twenty-three *AUXIN RESPONSE FACTORs* (*ARFs*) and IAA-responsive genes listed in TAIR were mined from the BAP-treated microarray data. Those showing significant change after BAP treatment are listed (Table 3.19). Of the twenty members of the auxin-responsive *GH3* family in *Arabidopsis* (Takase et al., 2003), ten were not represented or A(bsent) on the arrays. *GH3-12* had one of the highest increases (mean = 215) in transcript abundance in the BAP-treated samples. RT-PCR showed significant increase in transcript levels of this gene from 4 to 96 h after treatment with a return to control levels by 192 h (Figure 3.20).

The *ACS* family of genes serve in the rate-limiting first step of ethylene production (Yang and Hoffman, 1984; Chae et al., 2003). *ACS1* was not represented on the microarrays, and is thought to encode a non-functional *ACS* (Liang et al., 1995). *ACS4* is induced by auxin (Alonso and Ecker, 2001) and *ACS5* by cytokinins (Vogel et al., 1998). In this study, *ACS4* and *ACS5* were flagged A(bsent) on the microarrays. BAP treatment induced an increase in transcript levels of *ACS2* (Table 3.20). The *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE* (*ACCox*) group of genes encode enzymes responsible for the final step of ethylene production, which are also capable of stabilizing levels of ethylene biosynthesis (Kim and Yang, 1994). Two *ACCox-LIKE* genes had increased transcript levels in BAP-treated samples (Table 3.20).

A systemic pathogen response has been documented as co-regulated by ethylene and jasmonic acid through activation of specific members of the *ETHYLENE RESPONSE FACTOR* (*ERF*) family of 124 transcription factors (McGrath et al., 2005).

Table 3.18. Microarray data of genes with cytokinin oxidase function

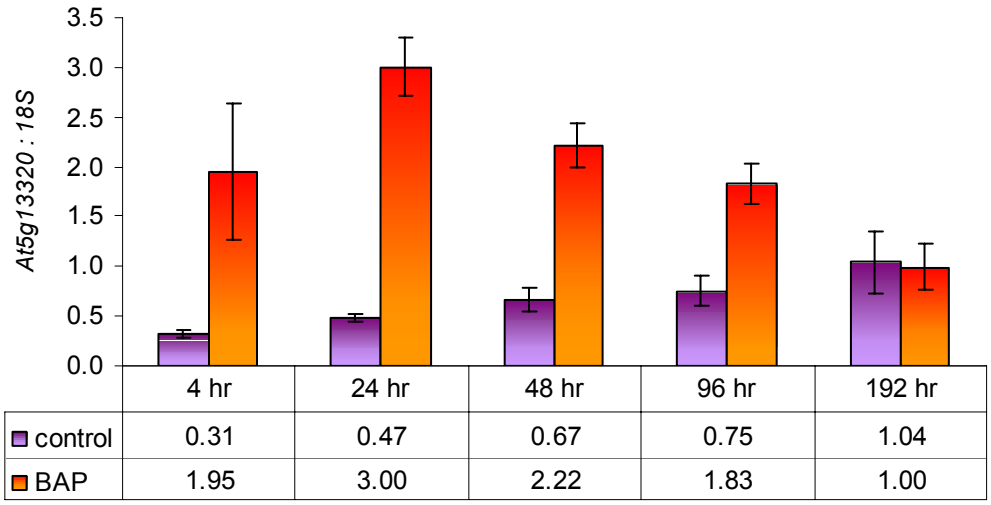
	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***CYTOKININ OXIDASE 3* (*CKX3*) At5g56970**												
22k	P	P	P	P	P	96.15	163.06	1072.19	2126.01	1184.68		
								11.15	22.11	12.32	**	***
***CKX4* At4g29740**												
22k	P	P	P	P	P	296.85	342.14	655.48	853.21	752.07		
								2.21	2.87	2.53	- -	***
8k	A	A	P	P	P	79.43	62.99	372.86	454.67	412.20		
								4.69	5.72	5.19	***	
***CKX5* At1g75450**												
22k	P	P	P	P	P	241.14	382.67	750.08	943.33	911.20		
								3.11	3.91	3.78	***	***

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

Table 3.19. Microarray data of auxin-responsive genes

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***AUXIN RESPONSE FACTOR 12* (*ARF12*) At1g34310**												
22k	P	P	P	P	P	3836.90	1552.37	910.01	2406.72	842.22		
								-4.22	-1.59	-4.56	- -	*
8k	A	A	A	A	A	16.29	4.77	2.18	3.68	0.82		
								-7.47	-4.42	-19.80	- -	
***GLYCINE HYPOCOTYL 3-4* (*GH3-4*) At1g59500**												
22k	A	P	P	P	P	10.68	91.43	174.19	268.89	230.52		
								16.30	25.17	21.58	**	***
***GH3-12* At5g13320**												
22k	A	A	P	P	P	6.10	3.77	1488.91	1470.03	3112.96		
								243.89	240.80	509.92	**	***
***GH3-14* At5g13360**												
22k	P	P	P	P	P	201.46	267.68	695.27	378.22	1433.96		
								3.45	1.88	7.12	*	**
At1g05680												
22k	A	A	P	P	P	85.47	80.12	466.27	331.68	1344.41		
								5.46	3.88	15.73	*	**
8k	A	P	P	P	P	94.37	101.17	192.61	148.85	454.17		
								2.04	1.58	4.81	*	
At2g26740												
22k	P	P	P	P	P	833.31	657.90	2097.08	1418.32	2392.35		
								2.52	1.70	2.87	***	**
8k	P	P	P	P	P	620.52	557.38	1604.09	1119.35	1743.44		
								2.59	1.80	2.81	***	
At2g33830												
22k	P	P	P	P	P	22183.50	2956.76	6039.72	15169.43	766.59		
								-3.67	-1.46	-28.94	- -	*
8k	P	P	P	P	P	20408.66	2695.27	4667.63	14431.41	964.28		
								-4.37	-1.41	-21.16	- -	
At3g14210												
22k	P	P	P	P	P	1813.14	849.23	460.26	474.25	274.09		
								-3.94	-3.82	-6.62	*	*
At5g35735												
22k	P	P	P	P	P	790.58	566.47	2193.19	1832.74	4346.65		
								2.77	2.32	5.50	**	**

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.



	4 hr	24 hr	48 hr	96 hr	192 hr
control	0.31	0.47	0.67	0.75	1.04
BAP	1.95	3.00	2.22	1.83	1.00

Figure 3.20. RT-PCR amplification of transcript levels of *GH3-12*, a member of the auxin-responsive GH3 family, in BAP-treated and control populations of *Arabidopsis,* over a flowering time course. Plant axes with leaves removed were harvested. Y-axis/table represents the mean ratio of the transcript levels of gene of interest to the 18S control (N = 3).

Table 3.20 Microarray data of genes associated with ethylene biosynthesis and response

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2 (ACS2)										**At1g01480**		
22k	A	A	P	P	P	9.16	14.14	478.28	400.38	1721.96		
								52.23	43.72	188.04	*	**
8k	A	A	P	A	P	17.65	19.09	42.88	26.53	102.85		
								2.43	1.50	5.83	--	
8k	A	A	P	P	P	55.67	53.45	190.43	166.54	752.01		
								3.42	2.99	13.51	*	
ACS5	**At5g65800**											
22k	A	A	A	A	A	9.16	9.43	4.51	22.16	10.29		
								-2.03	2.42	1.12	--	--
8k	A	A	A	A	A	50.24	23.86	5.09	40.53	4.11		
								-9.87	-1.24	-12.21	--	
ACS6	**At4g11280**											
22k	P	P	P	P	P	666.19	666.38	1421.33	1110.28	1824.21		
								2.13	1.67	2.74	--	***
8k	P	P	P	P	P	943.68	598.42	1669.50	1033.87	2082.42		
								1.77	1.10	2.21	*	
ACCox-LIKE	**At5g43450**											
22k	A	A	P	P	P	96.15	57.50	400.95	384.87	576.61		
								4.17	4.00	6.00	***	***
ACCox-LIKE	**At1g03400**											
22k	P	P	P	P	P	206.80	215.84	564.63	658.93	760.54		
								2.73	3.19	3.68	***	***
8k	A	A	P	P	P	53.63	15.27	133.01	255.70	116.83		
								2.48	4.77	2.18	*	
ETHYLENE RESPONSIVE FACTOR 1 (ERF1)										**At4g17500**		
22k	P	P	P	P	P	354.08	229.04	1487.40	1044.54	2363.92		
								4.20	2.95	6.68	**	**
8k	P	A	P	P	P	110.66	167.98	797.32	439.93	1193.01		
								7.21	3.98	10.78	**	
ERF2	**At5g47220**											
22k	P	A	P	P	P	242.67	125.36	572.89	575.46	583.26		
								2.36	2.37	2.40	***	--
8k	A	P	P	P	P	102.52	79.22	340.15	445.82	363.66		
								3.32	4.35	3.55	***	
MULTIPROTEIN BRIDGING FACTOR 1c (MBF1c)										**At3g24500**		
22k	P	P	P	P	P	307.53	525.94	1532.45	305.83	3426.38		
								4.98	-1.01	11.14	*	*
Ethylene-related enzyme	**At5g20400**											
22k	A	P	P	P	P	112.94	194.16	329.62	373.05	490.69		
								2.92	3.30	4.34	**	***
Unknown protein	**At4g39675**											
22k	P	P	A	P	P	1088.95	960.45	12.01	538.52	175.46		
								-90.65	-2.02	-6.21	**	--

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.

BAP induced a significant increase in transcript levels of two members, *ERF1* and *ERF2* (Table 3.20).

A group of three *MULTIPROTEIN BRIDGING FACTORs* (*MBF*) serve as transcriptional coactivators connected to an ethylene-response signal transduction pathway (Tsuda et al., 2004). *MBF1c* had significantly higher transcript levels in BAP-treated plants (Table 3.20).

A long ethylene-responsive motif, 5'-TAAGAGCCGCC-3', has been identified in ethylene-inducible pathogenesis-related genes in *Nicotianna spp* (Ohme-Takagi and Shinshi, 1995). This sequence was sought in the upstream regulatory region of all 653 significant BAP-responsive genes. It only appeared in the 1000 bp upstream sequence of At4g39675. This gene of unknown function had significantly decreased transcript abundance in BAP-treated plants (Table 3.20). Meta Analyzer indicated that this uncharacterized gene is strongly induced by ethylene and repressed by zeatin and light.

The transcriptomic data showed BAP induced changes in transcript levels of numerous genes functioning in defense (section 3.4.2). As salicylic acid is integral to regulation of systemic and localized defense responses in plants, genes attributed to its biosynthesis (Wildermuth et al., 2001) were mined from the data. *SALICYLIC ACID INDUCTION DEFICIENT 1* (*SID1*) and *SID2 = ISOCHORISMATE SYNTHASE I* (*ISC1*) had significantly higher transcript levels (Table 3.21). *PR1*, *PR2* and *PR5*, markers of systemic acquired resistance, also showed significantly higher levels (Table 3.14). *ISC1* and *PR5* loci are in close proximity on chromosome 1 (section 3.5.4).

PHENYLALANINE AMMONIA-LYASE (*PAL*) genes have also been described as serving in a salicylic acid biosynthetic pathway (Lee et al., 1995). However, this was challenged by *PAL* gene expression being lower in the *cdr1-D* mutant than wild type, despite increased salicylic acid levels and constitutive expression of inducible defense responses in the mutant (Suzuki et al., 2004a). In BAP-treated samples, transcript levels of *PAL1* and *PAL2* were not significantly increased, whereas *PAL3* had decreased levels (Table 3.21).

Table 3.21. Microarray data of genes associated with salicylic acid, gibberellic acid, and jasmonic acid biosynthesis and response

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
Salicylic acid												
***SALICYLIC ACID IINDUCTION DEFICIENT 1* (*SID1*) At4g39030**												
22k	P	P	P	P	P	250.30	280.88	1094.72	1577.89	1679.61		
								4.37	6.30	6.71	***	***
8k	P	P	P	P	P	35.30	92.58	359.78	614.57	703.46		
								10.19	17.41	19.93	***	
***SID2* (=*ICS1*) At1g74710**												
22k	P	P	P	P	P	281.59	246.00	2026.50	2993.99	3209.16		
								7.20	10.63	11.40	***	***
***PHENYLALANINE AMMONIA-LYASE 1* (*PAL1*) At2g37040**												
22k	P	P	P	P	P	2158.06	3865.38	5262.60	2441.44	5465.99		
								2.44	1.13	2.53	- -	- -
8k	P	P	P	P	P	978.31	1445.94	2250.96	1378.74	2867.33		
								2.30	1.41	2.93	- -	
***PAL2* At3g53260**												
22k	P	P	P	P	P	862.31	1674.36	658.19	1382.53	1295.06		
								1.94	-1.31	1.60	- -	- -
8k	P	P	P	P	P	844.56	843.84	517.30	902.57	764.49		
								-1.00	-1.63	1.07	- -	
***PAL3* At5g04230**												
22k	P	P	P	P	P	610.49	589.09	277.06	295.48	201.48		
								-2.20	-2.07	-3.03	- -	- -
8k	P	P	P	P	A	169.73	167.02	72.68	57.48	60.06		
								-2.34	-2.95	-2.83	***	
Gibberellic Acid												
***GAST1 PROTEIN HOMOLOG 1* (*GASA1*) At1g75750**												
22k	P	P	P	P	P	3495.03	2149.00	724.56	808.89	712.74		
								-4.82	-4.32	-4.90	***	*
8k	P	P	P	P	P	3966.18	2988.28	969.58	962.39	1002.13		
								-4.09	-4.12	-3.96	***	
Jasmonic Acid												
lipoxygenase, jasmonatesynthesis-degradation At1g72520												
22k	A	A	P	P	P	127.44	194.16	339.38	266.67	723.03		
								2.66	2.09	5.67	*	**
similarity to jasmonate-inducible protein At5g48180												
22k	P	P	P	P	P	753.19	951.97	1924.39	1699.77	6029.29		
								2.56	2.26	8.01	*	- -

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.

GA REQUIRING 2 (*GA2*) encodes a protein with ent-kaurene synthase B activity, catalyzing the second step of the gibberellic acid biosynthetic pathway, while *GA5* encodes gibberellin 20-oxidase, located further downstream in the pathway (Otsuka et al., 2004). *GA2* maintained steady state transcript levels and *GA5* was flagged A(bsent) in BAP-treated data. The *GA-STIMULATED* (*GASA*) family of five genes in *Arabidopsis* is related to *GA-STIMULATED TRANSCRIPT 1* (*GAST1*) in tomato. *GASA1*, which is normally strongly expressed in flower buds and young siliques and potentially has a regulatory role in reproduction (Herzog et al., 1995), had almost 5-fold lower transcript abundance in BAP-treated plants (Table 3.21). Eleven additional genes associated with a gibberellic acid response mined from the data were flagged A(bsent) or maintained steady state transcript levels. *SPINDLY* (*SPY*) encodes a signal transduction protein documented as responsive to cytokinins while repressing gibberellic acid signalling (Greenboim-Wainberg et al., 2005). *SPY* transcripts were not significantly altered by BAP.

DEFECTIVE IN ANTHER DEHISCENCE 1 (*DAD1*) catalyzes the initial step of jasmonic acid biosynthesis. *ALLENE OXIDE SYNTHASEs* (*AOSs*), *DELAYED DEHISCENCE 1* (*DDE1*), and *ALLENE OXIDE CYCLASEs* (*AOC*) are also essential to jasmonic acid production and upregulated by stresses such as wounding (Stenzel et al., 2003). These four genes maintained steady state transcript levels in BAP-treated populations. Of twenty-two genes associated with jasmonate responses mined from the data, only two, At1g72520 and At5g48180, showed a significant increase in transcript levels in BAP-treated samples (Table 3.21).

3.5.2 Kinases

The numerous families of receptor kinases identified in the *Arabidopsis* genome serve diverse and essential roles in developmental processes. In this study, genes encoding histidine kinases of two-component systems, CDPKs, MPKs, and LRR-kinases, such as *CLV1* (section 2.3.3) were mined from the BAP-treated data.

3.5.2.1 Two-component Systems

Fifty-four genes have been identified in the *Arabidopsis* genome as encoding

elements of two-component systems (Hwang et al., 2002). In the BAP-treated samples, five cytokinin-responsive (*AHK1-5*), five ethylene receptors (*ETR1, ETR2, EIN4, ERS1,* and *ERS2*), and five photoreceptors (*PHYA-E*) were not significantly affected at the transcript level, except *AHK2* showed a slight decrease (Table 3.22). Encoding intermediate proteins in two-component pathways (Suzuki et al., 2000b), five *HISTIDINE PHOSPHOTRANSFER PROTEINS* (*AHP1-5*) also maintained steady state transcript levels in BAP-treated populations.

Serving downstream of AHKs, the thirty-two members of the *ARR* gene family in *Arabidopsis* have been divided into three subfamilies: eleven A-type *ARRs*, twelve B-type *ARRs*, and nine *APRRs* (Hwang et al., 2002). Six members of the A-type subfamily showed significant but varied increases in transcript levels in BAP-treated plants (Table 3.22). *ARR5* / *ARR6* and *ARR7* / *ARR15*, gene duplicates within the family, are expressed in meristematic tissues (D'Agostino and Kieber, 1999; Leibfried et al., 2005). Transcript levels of the B-type *ARRs* were unaffected by BAP treatments. *APRR9* was not recognized as significant by SAM® as three of the samples had A(bsent) flags; however, the 8k data and KDE® suggest this gene may show a significant increase in transcript levels in two of the BAP-treated replicates (section 3.6.3).

3.5.2.2 *CDPKs* and *MPKs*

Of the 34 *CDPK/CPK* genes listed in TAIR, two, *CPK6* and *CPK29*, showed a significant increase in transcript levels with BAP treatment (Table 3.23). The *Arabidopsis* genome encodes 57 calmodulin (CaM) and CaM-like proteins; several genes encoding proteins associated with Ca^{2+} binding and calmodulin-binding had significantly increased transcript levels in BAP-treated samples (Table 3.23). Relatively little is known about the physiological function of these potentially important components of plant Ca^{2+} signal transduction (McCormack et al., 2005). BAP treatment also significantly increased transcript levels of *TCH3*, encoding a calmodulin-like protein with six potential Ca^{2+}-binding domains. *TCH3* is expressed in growing tissues and induced by touch, auxin, and darkness (Antosiewicz et al., 1995). Meta Analyzer data indicated that *TCH3* expression is not affected by zeatin and is downregulated by SA, ethylene, and senescence.

Table 3.22. Microarray data of elements of two-component systems

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***Arabidopsis HISTIDINE KINASE 2* (*AHK2*) At5g35750**												
22k	P	P	P	P	P	425.05	314.81	150.92 -2.82	224.57 -1.89	108.91 -3.90	- -	*
***Arabidopsis RESPONSE REGULATOR 4* (*ARR4*) At1g10470**												
22k	P	P	P	P	P	1988.66	3119.82	4961.52 2.49	6259.10 3.15	7181.29 3.61	- -	***
8k	P	P	P	P	P	1405.34	2031.95	2857.85 2.03	3680.07 2.62	3833.26 2.73	- -	
***ARR5* At3g48100**												
22k	P	P	P	P	P	592.17	1102.78	2110.60 3.56	2900.18 4.90	3118.41 5.27	***	***
***ARR6* At5g62920**												
22k	P	P	P	P	P	283.88	487.30	660.73 2.33	807.41 2.84	689.15 2.43	- -	***
***ARR7* At1g19050**												
22k	P	P	P	P	P	2113.04	2374.27	5133.46 2.43	5409.58 2.56	4443.46 2.10	- -	***
8k	P	P	P	P	P	323.84	318.77	622.16 1.92	1059.66 3.27	947.82 2.93	**	
***ARR15* At1g74890**												
22k	P	P	P	P	P	178.57	387.39	749.33 4.20	826.62 4.63	551.20 3.09	**	***
***ARR16* At2g40670**												
22k	P	P	P	P	P	365.53	527.83	1783.98 4.88	2418.54 6.62	2408.08 6.59	***	***
8k	P	P	P	P	P	106.59	93.53	169.35 1.59	502.56 4.71	459.10 4.31	*	
***Arabidopsis PSEUDO-RESPONSE REGULATOR 9* (*APRR9*) At2g46790**												
22k	A	A	P	A	P	68.68	17.91	185.46 2.70	66.48 -1.03	293.45 4.27	- -	- -
8k	A	P	P	A	P	70.61	125.03	195.51 2.77	59.69 -1.18	216.39 3.06	- -	

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased, blue is decreased transcript levels.

Table 3.23. Microarray data of *CDPKs* and genes associated with Ca^{2+} response

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***CPK6* = *CDPK3* At2g17290**												
22k	P	P	P	P	P	208.33	225.27	424.97	558.47	1069.12		
								2.04	2.68	5.13	*	**
8k	P	P	P	P	P	179.23	244.33	507.32	610.15	1032.57		
								2.83	3.40	5.76	- -	
***CPK29* At1g76040**												
22k	P	P	P	P	P	118.28	93.31	364.16	571.02	490.09		
								3.08	4.83	4.14	***	***
***TOUCH 3* (*TCH3*) At2g41100**												
22k	P	P	P	P	P	3924.66	2738.09	12119.98	16360.23	15302.22		
								3.09	4.17	3.90	***	***
8k	P	P	P	P	P	12901.97	11201.98	28157.71	29568.03	29558.62		
								2.18	2.29	2.29	- -	
calcium ion binding At1g76650												
22k	P	P	P	P	P	107.60	80.12	443.74	209.06	760.54		
								4.12	1.94	7.07	*	**
calcium ion binding At2g46600												
22k	P	P	P	P	P	1654.41	1686.21	5645.53	7014.80	4687.29		
								3.41	4.24	2.83	***	***
calcium ion binding At3g01830												
22k	A	P	P	P	A	6.10	28.28	155.42	217.92	287.40		
								25.46	35.70	47.08	**	***
calcium ion binding At3g47480												
22k	A	A	P	P	P	41.97	12.25	929.53	1148.70	1777.02		
								22.15	27.37	42.34	***	***
calcium ion binding At5g39670												
22k	P	P	P	P	P	150.33	176.26	1193.83	1625.16	1497.49		
								7.94	10.81	9.96	***	***
calcium ion binding At5g42380												
22k	A	P	P	P	A	9.16	2.83	394.19	181.72	1157.45		
								43.05	19.84	126.40	*	**
calcium ion binding At5g49480												
22k	P	P	P	P	P	502.12	1360.09	2369.64	977.31	4381.74		
								4.72	1.95	8.73	*	**
calcium ion storage At1g08450												
22k	P	P	P	P	P	1384.27	1490.16	3264.63	5432.48	4025.98		
								2.36	3.92	2.91	***	***
8k	P	P	P	P	P	549.92	479.12	1346.80	2008.78	1705.59		
								2.45	3.65	3.10	***	
calmodulin-like protein At2g41410												
22k	P	P	P	P	P	1454.48	1970.86	5896.31	6134.25	6826.74		
								4.05	4.22	4.69	***	***
8k	P	P	P	P	P	1376.15	1304.69	4459.76	4859.84	5039.43		
								3.24	3.53	3.66	***	

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

In all eukaryotic organisms, MPK cascades serve as mediators of diverse extracellular signals. In plants, MAPKKKs, MKKs and MPKs are involved in stress responses, hormone regulation, and developmental processes (Mizoguchi et al., 1994). Compared to six genes documented in yeast and 13 in mammals (Meskiene and Hirt, 2000), TAIR lists 113 genes encoding proteins with MPK function in *Arabidopsis*. In BAP-treated data only two, *MPK11* and *ZIK8*, showed a significant change in transcript abundance (Table 3.24). Meta Analyzer suggested hormone regulation of *MPK11* and *ZIK8* is not coordinated. The database also showed that *MPK11* is upregulated by salicylic acid and repressed by brassinolide.

3.5.3 Transcription Factors

The *Arabidopsis* genome includes numerous transcription factor families; extensive information is available at the Database of *Arabidopsis* Transcription Factors (DATF) http://datf.cbi.pku.edu.cn/. BAP significantly altered transcript abundance of members of several families; however, this sample may be conservative as small changes in expression (i.e. below 2.5-fold) of transcription factors may achieve significant change in biological processes. Analysis of the impact of BAP on transcription factors was limited by typically low expression levels, i.e. A(bsent) flags, of hybridization in microarray samples.

Members of the APETALA2/ETHYLENE RESPONSE ELEMENT BINDING PROTEIN (AP2/EREBP)-domain family are distinguished by a domain of approximately 70 amino acids termed the AP2 repeat (Okamuro et al., 1997; Alonso et al., 2003). The 146 transcription factors in this family were mined from the BAP-treated data; however, nine were not represented on the GeneChips®. Ten were identified as responding significantly to BAP treatment (Table 3.25). Meta Analyzer showed that seven of these ten are upregulated by ethylene.

Named for a domain first identified in the avian myeloblastosis virus, the large MYB-domain transcription factor family in *Arabidopsis* functions in secondary metabolism, disease resistance, stress responses, and hormone regulation (Meissner et al., 1999). Several plant-specific processes have been linked to MYB regulation,

Table 3.24. Microarray data of components of MPK cascades

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
MITOGEN-ACTIVATED PROTEIN KINASE 11 **(*MPK11*) At1g01560**												
22k	P	P	P	P	P	132.02	134.78	637.46	644.16	965.65		
								4.83	4.88	7.31	***	***
ZIK8 **At5g55560**												
22k	P	P	P	P	P	186.20	129.13	433.23	347.93	881.55		
								2.33	1.87	4.73	*	**

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); red is increased transcript levels.

Table 3.25. Microarray data of AP2/EREBP-domain transcription factors

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
ETHYLENE RESPONSIVE FACTOR 1 (ERF1)								**At4g17500^**				
22k	P	P	P	P	P	354.08	229.04	1487.40	1044.54	2363.92		
								4.20	2.95	6.68	**	**
8k	A	A	P	P	P	110.66	167.98	797.32	439.93	1193.01		
								7.21	3.98	10.78	**	
ERF2	**At5g47220^**											
22k	P	A	P	P	P	242.67	125.36	572.89	575.46	583.26		
								2.36	2.37	2.40	***	- -
8k	A	P	P	P	P	102.52	79.22	340.15	445.82	363.66		
								3.32	4.35	3.55	***	
At1g22985												
22k	A	P	P	P	P	239.62	174.37	450.50	357.54	788.98		
								1.88	1.49	3.29	*	- -
At2g38340												
22k	A	A	P	P	P	5.34	1.89	337.88	231.22	1553.76		
								63.25	43.28	290.87	*	**
8k	A	A	P	P	P	52.28	17.18	178.80	114.96	966.75		
								3.42	2.20	18.49	*	
At3g25730^												
22k	P	P	P	P	P	128.96	291.25	339.38	217.18	421.72		
								2.63	1.68	3.27	- -	**
At3g50260												
22k	P	P	P	P	P	201.46	237.52	659.98	589.49	1349.86		
								3.28	2.93	6.70	*	**
At3g61630^												
22k	A	A	P	P	P	279.30	291.25	753.09	903.44	1089.08		
								2.70	3.23	3.90	***	***
At5g13330^												
22k	A	P	P	P	P	77.84	127.24	1391.30	1044.54	3299.32		
								17.87	13.42	42.39	*	**
At5g25190^												
22k	P	P	P	P	P	280.06	213.02	769.61	916.74	931.17		
								2.75	3.27	3.32	***	***
At5g65510^												
22k	A	A	P	P	P	7.63	5.90	81.09	86.43	93.18		
								10.63	11.33	12.21	*	***

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels. ^ denotes genes which Meta Analyzer indicates have transcript levels elevated by ethylene treatment.

including anthocyanin synthesis, trichome formation, and desiccation tolerance (Kirik et al., 1998). Of the 133 MYB-domain genes mined from the data, three showed significant increases in transcript levels in BAP-treated populations (Table 3.26). Meta Analyzer data did not indicate a regulation pattern for these three genes. The responses of MYB factors *CCA1* and *LHY* to BAP treatment are reported later (section 3.6.3).

Members of the WRKY superfamily of transcription factors, defined by a conserved amino acid sequence WRKYGQK at its N-terminal end and a binding preference for the W-box cis-element, appear to be involved in the regulation of processes unique to plants, including senescence, pathogen defense, and trichome development (Eulgem et al., 2000). Sixteen of the 72 WRKY factors showed significantly increased transcript levels after BAP treatment (Table 3.27). Meta Analyzer indicated all 16 are induced by pathogens and ozone, and that half of the 16, *WRKY6, 38, 40, 46, 53, 54, 70*, and *75*, are induced by salicylic acid. Only *WRKY54* showed a response to zeatin, and in this case with decreased levels. The promoter motif 5'-AGCCGCC-3', associated with ethylene regulation, is found 350 bases upstream in the regulatory region of *WRKY33*; Meta Analyzer reports ethylene induced this gene by over 2.5-fold. BAP treatment increased transcript levels of *WRKY33* (Table 3.27).

Exclusive to the plant kingdom, NO APICAL MERISTEM (NAM), ARABIDOPSIS TRANSCRIPTIONAL ACTIVATOR FAMILY 1 (ATAF1), and CUP-SHAPED COTYLEDON 2 (CUC2) define the conserved domain of the NAC transcription factor family, functioning in formation and maintenance of apical meristems, floral morphogenesis, defense, and response to blue light (Jiao et al., 2003; Ooka et al., 2003). Of the 133 genes in the NAC family, 125 were represented on the GeneChips®, and 16 showed significantly increased transcript abundance in BAP-treated samples (Table 3.28). This included *ATAF1*, a gene induced by wounding, dehydration, salinity, ABA, and reactive oxygen species (Souer et al., 1996; Fujita et al., 2004), and *NAP*, a flower development gene (Sablowski and Meyerowitz, 1998). Meta Analyzer indicated that 12 of the 16 are upregulated by more than 2.5-fold by *P. syringae*.

Table 3.26. Microarray data of MYB-domain transcription factors

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
MYB13 **At1g06180**												
22k	P	P	P	P	P	252.59	538.19	680.26	537.04	833.75		
								2.69	2.13	3.30	- -	***
8k	P	P	P	P	P	183.98	256.74	630.15	390.56	803.02		
								3.43	2.12	4.36	**	
MYB62 **At1g68320**												
22k	A	A	P	P	P	80.13	67.86	166.69	167.69	262.59		
								2.08	2.09	3.28	**	- -
MYB-like **At1g70000**												
22k	A	A	P	P	P	167.12	226.21	864.21	614.61	1114.50		
								5.17	3.68	6.67	***	**
8k	A	A	P	P	P	103.87	51.54	159.90	324.24	591.57		
								1.54	3.12	5.70	*	

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

Table 3.27. Microarray data of WRKY-domain transcription factors

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
WRKY6 **At1g62300**												
22k	P	A	P	P	P	160.25	167.77	538.35	475.73	1312.35		
								3.36	2.97	8.19	*	**
8k	A	P	P	P	P	197.56	170.84	598.90	487.09	1331.23		
								3.03	2.47	6.74	*	
WRKY15 **At2g23320**												
22k	P	P	P	P	P	836.36	477.87	895.00	2091.29	1974.27		
								1.07	2.50	2.36	*	*
8k	P	P	P	P	P	931.46	577.42	1942.06	1740.55	2330.07		
								2.08	1.87	2.50	***	
WRKY18 **At4g31800**												
22k	P	P	P	P	P	393.00	290.30	1601.53	1415.37	2546.03		
								4.08	3.60	6.48	***	**
8k	A	P	P	P	P	114.74	120.26	141.00	159.17	100.38		
								1.23	1.39	-1.14	- -	
WRKY25 **At2g30250**												
22k	P	P	P	P	P	312.11	293.13	849.19	929.30	1815.74		
								2.72	2.98	5.82	**	**
8k	P	P	P	P	P	114.06	146.03	178.07	695.63	1559.14		
								1.56	6.10	13.67	*	
WRKY28 **At4g18170**												
22k	A	A	P	P	P	43.50	11.31	111.87	152.91	183.93		
								2.57	3.52	4.23	**	***
8k	A	A	A	P	P	14.94	4.77	51.60	74.43	145.63		
								3.46	4.98	9.75	- -	
WRKY31 **At4g22070**												
22k	A	P	P	P	P	88.52	137.61	295.83	115.98	524.58		
								3.34	1.31	5.93	*	- -
8k	A	A	P	A	P	41.41	80.17	249.30	25.05	624.48		
								6.02	-1.65	15.08	- -	
WRKY33 **At2g38470**												
22k	P	P	P	P	P	696.72	436.40	2042.27	1452.31	3299.32		
								2.93	2.08	4.74	**	**
8k	P	P	P	P	P	255.27	192.79	1083.69	848.91	2052.80		
								4.25	3.33	8.04	**	
WRKY38 **At5g22570**												
22k	P	P	P	P	P	109.89	146.09	1108.98	1091.81	750.86		
								10.09	9.94	6.83	***	***
WRKY40 **At1g80840**												
22k	P	A	P	P	P	433.44	261.08	1100.72	1044.54	1584.62		
								2.54	2.41	3.66	***	**

WRKY46	**At2g46400**											
22k	P	P	P	P	P	327.37	204.53	3408.04	3304.99	4242.58		
								10.41	10.10	12.96	***	***
WRKY48	**At5g49520**											
22k	A	A	P	P	P	95.39	186.62	481.29	236.39	922.09		
								5.05	2.48	9.67	*	**
WRKY53	**At4g23810**											
22k	P	P	P	P	P	241.90	180.03	763.60	1156.82	731.50		
								3.16	4.78	3.02	***	***
8k	P	P	P	P	P	154.11	77.31	490.60	759.74	454.99		
								3.18	4.93	2.95	***	
WRKY54	**At2g40750**											
22k	A	A	P	P	P	222.83	248.83	917.52	746.10	542.73		
								4.12	3.35	2.44	***	***
WRKY70	**At3g56400**											
22k	P	P	P	P	P	767.69	535.37	4711.49	5179.84	5166.49		
								6.14	6.75	6.73	***	***
WRKY75	**At5g13080**											
22k	A	P	P	P	P	10.68	78.23	486.54	257.81	1718.94		
								45.54	24.13	160.90	*	**

Flags: P(resent); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

Table 3.28. Microarray data of NAC family transcription factors

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR 1 (ATAF1) At1g01720												
22k	P	P	P	P	P	196.88	194.16	362.65	365.66	887.00		
								1.84	1.86	4.51	*	--
NAC-LIKE, ACTIVATED BY AP3/PI (NAP) At1g69490												
22k	A	A	P	P	P	154.15	87.66	238.01	464.65	870.06		
								1.54	3.01	5.64	*	--
At1g02220												
22k	A	A	P	P	P	51.89	28.28	1219.36	701.78	2785.03		
								23.50	13.52	53.67	*	**
At1g34180												
22k	A	A	P	P	P	61.05	77.29	203.48	340.55	285.58		
								3.33	5.58	4.68	**	***
At1g52890												
22k	P	A	P	P	P	141.17	61.27	482.79	911.57	2274.37		
								3.42	6.46	16.11	*	--
At2g17040												
22k	P	A	P	P	P	144.23	137.61	1121.75	1682.04	1085.45		
								7.78	11.66	7.53	***	***
8k	A	A	P	P	P	28.51	36.27	254.39	333.08	270.69		
								8.92	11.68	9.49	--	
At2g43000												
22k	P	A	P	P	P	163.30	91.43	313.10	351.63	772.04		
								1.92	2.15	4.73	*	**
At3g04060												
22k	A	A	P	P	P	75.55	51.84	283.06	172.12	565.72		
								3.75	2.28	7.49	*	**
At3g04070												
22k	P	P	P	P	P	282.35	243.18	492.55	452.09	1317.19		
								1.74	1.60	4.67	*	--
At3g10500												
22k	P	P	P	P	P	576.91	853.00	1772.72	2207.27	2853.40		
								3.07	3.83	4.95	***	**
At3g15500												
22k	A	A	M	M	P	93.86	96.14	174.94	145.53	431.40		
								1.86	1.55	4.60	*	--
At3g49530												
22k	P	P	P	P	P	675.35	475.04	1242.63	1751.48	3050.04		
								1.84	2.59	4.52	**	**
At4g27410												
22k	P	P	P	P	P	476.94	196.05	616.44	586.54	1471.47		
								1.29	1.23	3.09	*	*

At5g13180												
22k	P	P	P	P	P	1668.15	1422.30	3601.76	3733.44	5340.14		
								2.16	2.24	3.20	***	**
At5g18270												
22k	A	P	P	P	P	178.57	213.02	370.16	302.13	929.35		
								2.07	1.69	5.20	*	*
At5g63790												
22k	P	P	P	P	P	550.20	732.36	1421.33	1423.50	3564.93		
								2.58	2.59	6.48	*	**
8k	P	P	P	P	P	406.67	369.36	1374.42	1719.18	2374.50		
								3.38	4.23	5.84	***	

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

3.5.4 Co-Regulation of Significant Genes

Microarray technology facilitates simultaneous analysis of the transcript abundance of thousands of genes in response to specific conditions, potentially revealing coincidental gene expression patterns that can increase our understanding of functional genomics. The microarray data in combination with the *Arabidopsis* Chromosome Mapping Tool supplied by TAIR were ideal for an investigation of potential co-regulation by BAP. In the present study, clusters and patterns within the 653 significant genes were sought as potential indicators of co-regulation based on coincidental BAP-altered transcript levels, biological function, and chromosomal position. This identified 24 genes of interest, in clusters of two to five (Figure 3.21; Table 3.29). Chromosome I: three tandem genes encoding FAD-binding domain electron carrier proteins, At1g26380, At1g26390, At1g26420; two tandem genes associated with stress response, *ICS1* (At1g74710), a component of salicylic acid biosynthesis, and *PR5* (At1g75040), encoding a marker protein of systemic stress response; a tandem pair of genes functioning in development, *CLV1* (At1g75820), regulating shoot meristem activity, and *GASA1* (At1g75750), active in flower development. Chromosome IV: four genes in two tandem pairs on opposite strands encoding proteins with kinase function (At4g23140, At4g23150, At4g23260, At4g23320); the divergent genes *HEAT SHOCK FACTOR 4* (*HSF4*) (At4g36990) and one encoding an "unknown protein" (At4g37290). Chromosome V: two tandem genes of an auxin-responsive family, *GH3-12* (At5g13320) and *GH3-14* (At5g13360); tandem cold-inducible genes, *KIN1* (At5g15960) and *KIN2* (At5g15970). The latter pair showed significance by KDE® criteria rather than SAM® due to altered expression in the C2 array/T_1 generation.

Many more genes shared BAP-induced transcript abundance patterns, but these fell in the lower range of fold changes, where coincidence rather than biological significance was likely responsible. However, a pair of LRR protein kinases that are "light responsive" (At1g51800, At1g51890) and five tandem disease resistance genes with consecutive chromosome position (At1g72890, At1g72900, At1g72920, At1g72930, At1g72940) were notable within this group because of the proposed connection between cytokinins and light, and pathogens (section 3.4).

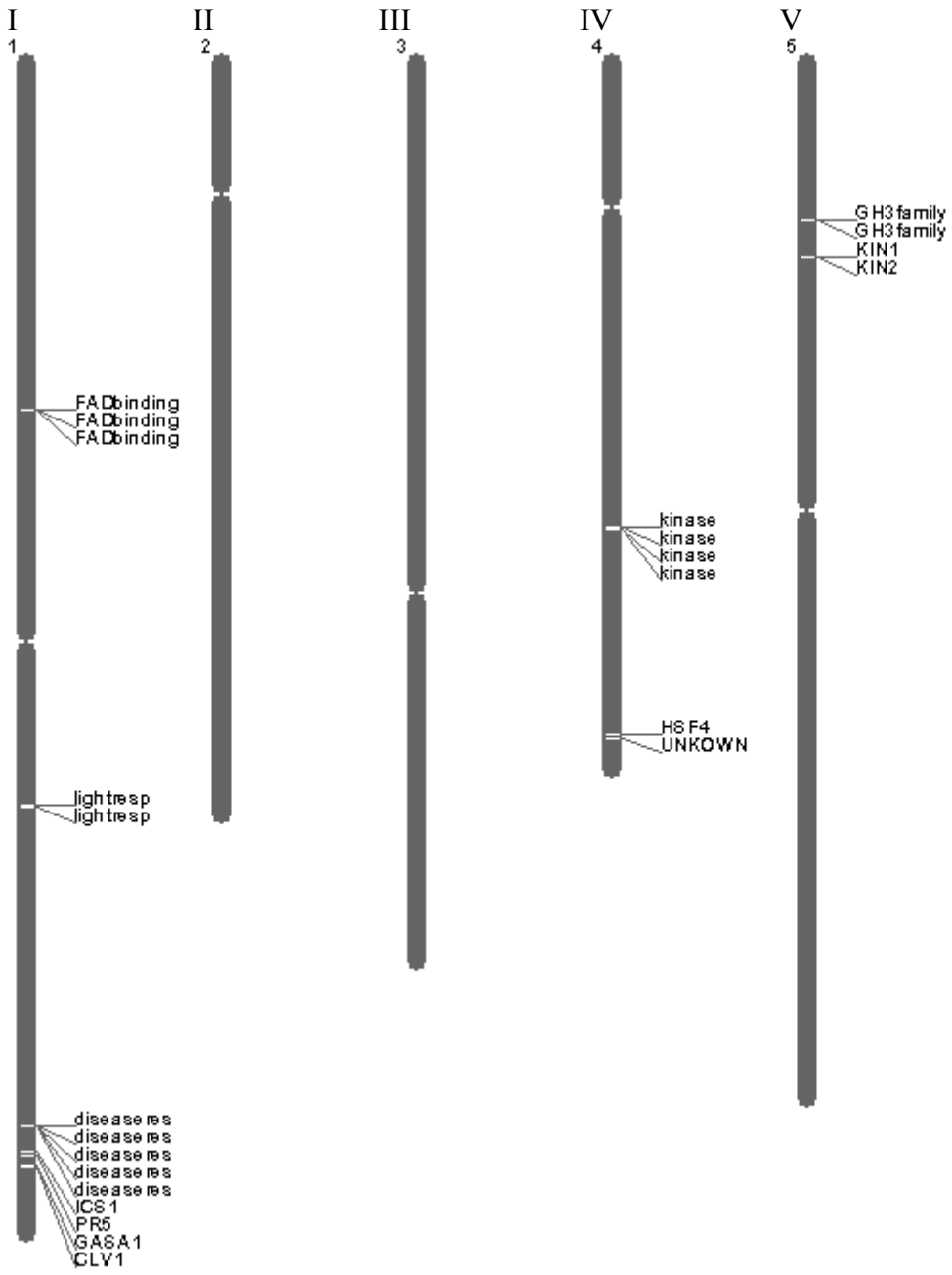


Figure 3.21. Chromosome map of 24 genes sharing coincident chromosome position, biological function, and BAP-altered transcript abundance (Table 3.29).

Table 3.29. Microarray data of 24 genes sharing chromosome proximity, biological function, and response to BAP-treatment

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	Mean
Chromosome I											
electron transfer flavoprotein At1g26380											
22k	A	P	P	P	P	67.15	134.78	1579.76	867.99	3472.36	
								23.52	12.93	51.71	29.39
electron transfer flavoprotein At1g26390											
22k	A	P	P	P	P	46.55	160.23	1805.01	525.96	3633.30	
								38.78	11.30	78.05	42.71
electron transfer flavoprotein At1g26420											
22k	A	A	P	P	P	3.05	5.66	557.12	290.31	1022.53	
								182.52	95.11	334.99	204.20
light repressible receptor protein kinase At1g51800											
22k	A	P	P	P	P	125.91	270.51	430.23	463.17	609.89	
								3.42	3.68	4.84	3.98
light repressible receptor protein kinase At1g51890											
22k	A	P	P	P	P	132.78	158.35	512.07	316.17	963.23	
								3.86	2.38	7.25	4.50
disease resistance protein At1g72890											
22k	A	A	P	P	P	93.10	34.87	443.74	549.60	1152.61	
								4.77	5.90	12.38	7.68
virus resistance protein At1g72900											
22k	P	P	P	P	P	148.81	161.18	623.94	339.81	689.75	
								4.19	2.28	4.64	3.70
disease resistance protein At1g72910											
not represented on arrays											
virus resistance protein At1g72920											
22k	P	P	P	P	P	95.39	107.45	401.70	249.68	203.30	
								4.21	2.62	2.13	2.99
flax rust resistance protein At1g72930											
22k	P	P	P	P	P	1203.42	1933.16	4303.04	4603.65	3720.43	
								3.58	3.83	3.09	3.50
8k	P	P	P	P	P	936.21	1117.62	2511.89	2893.80	2504.49	
								2.68	3.09	2.68	
disease resistance protein At1g72940											
22k	P	P	P	P	P	132.02	172.49	444.49	352.37	426.56	
								3.37	2.67	3.23	3.09

ISOCHORISMATE SYNTHASE (ICS1) At1g74710

22k	P	P	P	P	P	281.59	246.00	2026.50	2993.99	3209.16	
								7.20	10.63	11.40	9.74

PATHOGENESIS-RELATED (PR5) At1g75040

22k	M	P	P	P	P	279.30	261.08	2983.07	4969.31	3807.56	
								10.68	17.79	13.63	14.04
8k	P	P	P	P	P	510.54	379.86	4974.35	7348.35	5866.31	
								9.74	14.39	11.49	11.88

GAST1 PROTEIN HOMOLOG 1 (GASA1) At1g75750

22k	P	P	P	P	P	3495.03	2149.00	724.56	808.89	712.74	
								-4.82	-4.32	-4.90	-4.68
8k	P	P	P	P	P	3966.18	2988.28	969.58	962.39	1002.13	
								-4.09	-4.12	-3.96	-4.06

CLAVATA 1 (CLV1) At1g75820

22k	P	P	P	P	P	3446.95	1698.47	918.27	1067.44	626.83	
								-3.75	-3.23	-5.50	-4.16
8k	P	P	P	P	P	525.48	362.68	108.30	267.49	176.07	
								-4.85	-1.96	-2.98	-3.27

Chromosome IV

kinase protein At4g23140

22k	A	A	P	P	P	34.34	42.41	519.58	1020.16	882.76	
								15.13	29.71	25.71	23.51

kinase protein At4g23150

22k	A	A	P	P	P	6.10	50.90	461.01	613.13	641.35	
								75.52	100.43	105.06	93.67

kinase protein At4g23320

22k	A	A	P	P	P	22.13	38.64	203.48	186.16	208.14	
								9.19	8.41	9.41	9.00
8k	A	A	P	P	P	19.69	118.47	109.06	103.67	3.82	
								6.02	5.54	5.27	5.61

kinase protein At4g23260

22k	P	P	P	P	P	88.52	81.06	508.97	742.39	653.23	
								7.38	5.75	8.39	7.17
8k	A	A	P	P	P	23.76	2.86	109.06	297.84	180.25	
								7.59	4.59	12.53	8.24

HEAT SHOCK FACTOR 4 (HSF4) At4g36990

22k	P	P	P	P	P	257.93	422.26	2395.92	1441.96	3660.53	
								9.29	5.59	14.19	9.69
8k	P	P	P	P	P	351.67	608.92	2878.93	1898.25	4449.51	
								8.19	5.40	12.65	8.75

unknown protein At4g37290

	A	A	P	P	P	100.73	56.55	688.52	309.52	587.50	
								6.84	3.07	5.83	5.25

Chromosome V											
GH3-12											
22k	A	A	P	P	P	6.10	3.77	1488.91	1470.03	3112.96	
								243.89	240.80	509.92	331.53
GH3-14											
22k	P	P	P	P	P	201.46	267.68	695.27	378.22	1433.96	
								3.45	1.88	7.12	4.15
KIN1 **At5g15960**											
22k	P	P	P	P	P	11273.37	4540.24	4643.92	5209.39	2303.41	
								-2.43	-2.16	-4.89	-3.16
8k	P	P	P	P	P	13118.54	5247.38	5296.33	6330.69	2790.82	
								-2.48	-2.07	-4.70	-3.08
KIN2 **At5g15970**											
8k	P	P	P	P	P	11576.74	4659.46	4818.09	6486.18	3271.31	
								-2.40	-1.78	-3.54	-2.58

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. Means are of BAP-induced changes in transcript levels in R1-3 relative to C1.

3.5.4.1 Cis-elements

The 1000 bp up- and downstream regions of the 24 genes sharing loci proximity, biological function, and transcript response to BAP treatment (Table 3.29) were analysed for known cis-elements associated with transcription factor families (Table 3.30), and cis-elements associated with cytokinins, auxin, ethylene, and salicylic acid regulation, cold-acclimation, and circadian rhythms (Table 3.31). Important elements in cytokinin signal transduction, the transcription factors *ARR1* and *ARR2* optimally bind to the DNA sequence 5'-AGATT-3' in vitro (Sakai et al., 2000; Ross et al., 2004). This regulatory binding sequence has been shown to be active in BA responses in cucumber (Jin et al., 1998). This motif was found in the upstream region of 21 of the 24 genes analysed for co-regulation in the BAP-treated samples of this study, including five copies in *PR5*, a gene associated with the systemic defense response.

The reverse compliment of this cytokinin-linked cis-element is associated with the regulation of biological clock genes. The CCA1- binding/evening element 5'-aaAATATCT-3' (Harmer et al., 2000) is present in *KIN1* and *KIN2*, as well as other cold-acclimation genes, *COR15b*, *COR78*, and *COR413*, all of which had decreased transcript levels in the BAP-treated populations.

The G-box cis-element 5'-CACGTG-3' is associated with PHYA-responsive promoters, in association with light, pathogens, and redox changes (Giuliano et al., 1988; Menkens et al., 1995; Hudson and Quail, 2003). This element was found in the upstream sequences of *CLV1*, *KIN1*, *KIN2*, and At4g37290. As might be expected, the regulatory region of *KIN1* and *KIN2* also contained the dehydration responsive binding element (DREB) 5'-TACCGACAT-3' associated with cold-induced expression (Yamaguchi-Shinozaki and Shinozaki, 1994; Seki et al., 2001). Members of a subfamily of AP2/EREBP transcription factors bind to the core region of this element, 5'-CCGAC-3', which was found in 12 of the 24 genes of interest (Table 3.31). Due to the close association between cytokinins and ethylene, the ethylene-responsive motif 5'-AGCCGCC-3' (Bowman et al., 1992) was sought in the regulatory regions of the 24 genes of interest. A reverse complement was found 269 bases upstream of the start codon of *CLV1*. *ERFs* show binding affinity to the core of this sequence (Fujimoto et al., 2000); the ERF-domain 5'-GCC-3' box appears numerous times.

Table 3.30. Transcription factor binding domains in 24 genes of interest

	GCC AP2/ERERBP		(T/C)AAC(T/G)G MYB (Type I)		G(G/T)T(A/T)G(G/T)T MYB (Type II)		(T)TGAC(C/T) WRKY	
1000bp	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
At1g75820 *CLV1*	11	1	2	-	-	-	3	2
At1g75750 *GASA1*	1	8	1	-	-	-	-	-
At5g15960 *KIN1*	6	4	-	-	1	1	-	-
AT5g15970 *KIN2*	7	3	-	-	1	-	-	-
At1g26380 FADbinding	2	2	-	-	2	1	1	-
At1g26390 FADbinding	4	6	-	-	-	1	-	-
At1g26420 FADbinding	2	5	1	-	1	1	1	1
At1g74710 *ICS1*	4	4	1	1	-	-	-	-
At1g75040 *PR5*	5	5	-	1	-	2	-	-
At4g23140 kinase	4	5	2	-	-	1	5	-
At4g23150 kinase	4	4	-	1	-	-	-	1
At4g23320 kinase	7	8	-	-	-	1	1	-
At4g23260 kinase	3	7	-	1	-	3	2	2
At4g36990 *HSF4*	5	7	-	1	2	1	-	1
At4g37290 unknown	1	5	-	2	4	-	-	-
At1g51800 lightresp kinase	4	6	1	-	-	-	2	-
At1g51890 lightresp kinase	3	5	1	-	-	-	3	2

	GCC AP2/ERERBP		(T/C)AAC(T/G)G MYB (Type I)		G(G/T)T(A/T)G(G/T)T MYB (Type II)		(T)TGAC(C/T) WRKY	
1000bp	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
At5g13320 *GH3-12*	4	6	-	1	-	1	2	-
At5g13360 *GH3-14*	1	6	-	1	-	-	1	3
At1g72890 disease resistance	4	10	-	-	-	1	-	1
At1g72900 disease resistance	2	9	-	1	3	2	-	-
At1g72920 disease resistance	7	11	-	-	-	1	-	2
At1g72930 disease resistance	9	4	-	-	1	2	1	-
At1g72940 disease resistance	6	11	-	-	-	1	-	-

The occurrence of some published binding motifs 5'-3' associated with transcription factor families in the up- and downstream 1000 bp of 24 genes sharing similar chromosome position, biological function, and transcript abundance altered by BAP.

Table 3.31. Hormone and environmental response motifs in 24 genes of interest

	AGATT (cytokinin)		TGACG (salicylic acid)		CACGTG (light and pathogens)		CCGAC (cold)		aaAATATCT (circadian)	
1000bp	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
At1g75820 *CLV1*	-	3	1	-	1	-	-	-	-	-
At1g75750 *GASA1*	1	-	-	-	-	-	1	-	-	-
At5g15960 *KIN1*	2	-	-	-	2	-	1	2	1	2
AT5g15970 *KIN2*	1	5	-	1	3	-	2	1	1	1
At1g26380 FADbinding	1	1	2	-	-	-	2	2	-	2
At1g26390 FADbinding	-	-	-	1	-	-	-	-	-	-
At1g26420 FADbinding	2	3	-	1	-	-	1	2	-	2
At1g74710 *ICS1*	1	4	-	-	-	-	2	-	-	-
At1g75040 *PR5*	5	4	-	-	-	1	1	-	-	-
At4g23140 kinase	1	1	-	1	-	-	-	-	-	-
At4g23150 kinase	3	2	-	1	-	-	1	-	-	-
At4g23320 kinase	3	2	1	1	-	1	1	-	-	-
At4g23260 kinase	1	-	1	-	-	-	-	-	-	-
At4g36990 *HSF4*	3	1	-	-	-	-	1	-	1	-
At4g37290 unknown	1	7	-	1	1	1	-	1	-	1
At1g51800 lightresp kinase	3	1	-	1	-	-	-	-	-	-
At1g51890 lightresp kinase	3	1	-	-	-	-	-	1	-	1

	AGATT (cytokinin)		TGACG (salicylic acid)		CACGTG (light and pathogens)		CCGAC (cold)		aaAATATCT (circadian)	
1000bp	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
At5g13320 *GH3-12*	1	4	-	-	-	-	-	-	-	-
At5g13360 *GH3-14*	2	3	-	-	-	-	-	-	-	-
At1g72890 disease resistance	1	3	-	-	-	-	-	1	-	-
At1g72900 disease resistance	-	-	-	-	-	-	-	-	-	-
At1g72920 disease resistance	2	4	1	-	-	-	1	-	-	-
At1g72930 disease resistance	-	1	-	1	-	-	-	-	-	-
At1g72940 disease resistance	3	3	-	-	-	-	1	-	-	-

The occurrence of some published binding motifs 5'-3' associated with hormone, temperature, and circadian regulation in the up- and downstream 1000 bp of 24 genes sharing similar chromosome position, biological function, and transcript abundance altered by BAP.

The *activation sequence-1* (*as-1*) element 5'-TGACG-3' mediates induction by salicylic acid, responses to wounding, and is associated with auxin regulation (An et al., 1990; Strompen et al., 1998; Grüner et al., 2003). *As-1* was found in the upstream region of five of the 24 genes, *CLV1*, At1g26380, At4g23320, At4g23260, and At1g72920.

3.5.4.3 Putative Novel Cis-elements

Shared sequences in the regulatory regions of suspected co-expressed genes were sought as putative cis-elements (Table 3.32). The computer programs MEME and MAST (http://meme.nbcr.net/meme/website/meme.html) were used to analyse the upstream 1000 bp of *CLV1* and *GASA1*. The 21 base sequence 5'-TGTGGTGTTTTTCGTCTTTTC-3', with wobble, occurs five times in *CLV1* and three times in *GASA1*, with a probability of 0.5 and an e-value (product of p-values and occurrence) of 8.7.

The same programs were used to compare *ICS1* and *PR5* and found that a 29 base sequence, 5'-ATCTATTCGGATCTCATACTCGCAGATTA-3', with variation, occurred six times in *PR5* and once in *ICS1*, with a probability of 0.8 and an e-value 1.2. The sequence 5'-TATAAATGG-3' was found in the 1000 bp upstream sequences of clustered kinases At4g23140 and At4g23150 and it also occurs in the 3' UTR of *HSF4* and downstream of At4g37290, the gene encoding an unknown protein.

Combinations of the 24 genes of interest were also tested with the recently developed Motif Analysis tool offered by TAIR (Table 3.32). This tool identifies potentially novel 6mer sequences, lists the number of genes in the genomic set featuring the 6mer, and calculates the p-value for its occurrence in the genes of interest. The literature was then searched for described function of the generated 6mers. Four of the five disease response genes were found to share 5'-GCCGCC-3', a C-box cis-element associated with jasmonic acid regulated defense (Chakravarthy et al., 2003).

Table 3.32. Putative novel cis-elements responsive to BAP, as determined by Meme and Mast and the Motif Analysis tool at TAIR

	Putative novel cis-elements	**Occurrence in genome set**	**p-value**
CLV1 GASA1	TGTGGTGTTTTTCGTCTTTTC		
CLV1, *KIN1*, *KIN2*	GGCACC	1812 / 28088	2.68e-04
	TACGGG	2134 / 28088	4.39e-04
Three FAD elec. binding	CGTTGT	6926 / 28088	1.50e-02
	CGTATT	7408 / 28088	1.83e-02
Four receptor kinases	GTCTAG	5093 / 28088	1.08e-03
Four receptor and two light kinases	TCAACG	8287 / 28088	6.60e-04
ICS1, *PR5*	ATCTATTCGGATCTCATACTCGCAGATTA		
HSF4, unknown	TATAAATGG		
ICS1, *PR5, HSF4*, unknown	ACGATA	7655 / 28088	5.52e-03
ICS1, *HSF4*, unknown	GCACGA	3170 / 28088	5.10e-03
	CGACTC	3941 / 28088	9.50e-03
Five disease resistance	GCCGCC	2516 / 28088	2.93e-04
	ACGAAC	6442 / 28088	6.35e-04

The 24 genes of interest clustered by biological function, response to BAP, and chromosome proximity. Meme, Mast, and/or Motif Analysis tools were used to determine potentially novel cis-elements in the 1000 bp upstream of the Atg. The occurrence of each novel element in the same regulatory region of the 28,088 annotated *Arabidopsis* genes, along with the p-value associated with the rate of occurrence are listed, where applicable.

3.6 BAP-induced Epigenetic Inheritance

Seed harvested from the R1 population was grown to investigate inheritance of BAP-effects. Less than 50% of the seed germinated, so that most of the population needed to be harvested for the C2/T1 microarray sample. Floral and vegetative phenotypes of the remaining plants were recorded.

3.6.1 Inheritance of BAP-induced Phenotypes

T_1 plants produced aberrant floral phenotypes similar to BAP-treated plants (Table 3.33), but at a reduced rate (Figure 3.22). Inherited floral phenotypes included increased organ number, buds in the axil of sepals, forked stamens, and intermediary petaloid-stamen organs. In an experiment separate from the microarray populations, T_1 seed was collected from BAP-treated flowers/siliques with specific phenotypes, such as forked stamen filaments. A correlation between the BAP-treated phenotypes and subsequent T_1 phenotypes was not apparent. To test the cumulative affect of cytokinin treatments on the incidence of inheritance, three consecutive generations were treated with BAP. Counter intuitively; this reduced the rate of epigenetic variation in floral development (data not shown).

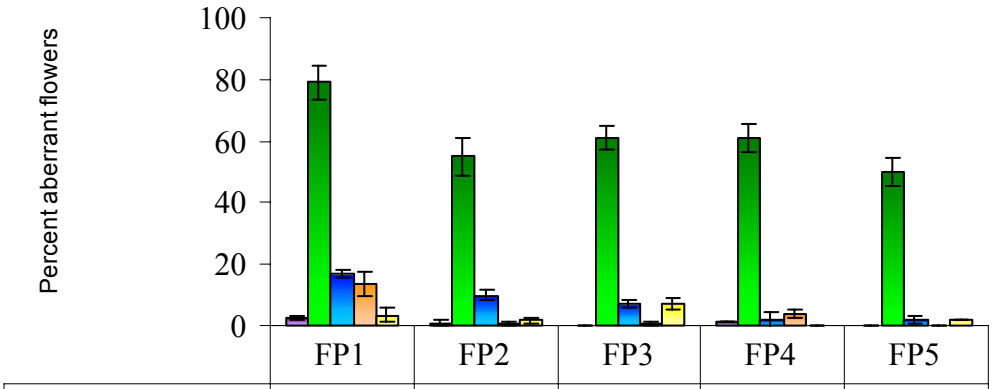
A conventional method for comparing flower timing is to count the number of rosette leaves at the time of bolting (Somers et al., 1998a; Wang and Tobin, 1998; Sheldon et al., 2000). For the present study, the average number of leaves for T_1 plants at time of bolting was 6.0 and controls 7.4 (N = 50), indicating precocious flowering in T_1 plants. T_0 were not documented as the BAP was applied after transition of the shoot meristem to reproductive function. A chi-square test of homogeneity at 0.005 rejected the null hypothesis; therefore the flowering was significantly earlier in the T_1 plants.

Plants of T_1 generations, descended from populations yielding the R1 and R3 samples, were measured for shoot length. After natural senescence and drying, the shoot of controls averaged 14.2 cm (range 11.5 to 20) and T_1 plants 17.0 cm (range 13.5 to 21); both groups N = 50 plants in one population. Again, a chi-square test of homogeneity indicated that there was a significant difference between the populations.

Table 3.33. Aberrant flower development in the T_1 generation of BAP-treated R1.

Chip	N	af	↑s	↓s	↑p	↓p	↑st	↓st	fst	pst	↑c
C1	685	1.8	0.6	0.0	0.6	0.0	0.5	12.6	0.1	0.0	0.0
R1	440	41.3	21.3	2.2	23.3	3.4	17.2	15.8	1.3	1.4	5.2
R2	425	53.2	15.2	2.5	23.4	1.1	13.7	16.4	0.5	1.1	3.4
R3	245	56.3	17.5	2.0	24.5	1.2	14.3	10.2	0.5	1.2	11.8
C2/T1	**205**	**13.1**	**2.0**	**1.2**	**3.6**	**1.0**	**1.4**	**22.3**	**2.3**	**0.5**	**1.8**

Microarray Chips: C1 control; R1-3 BAP-treated; C2/T1 second control also represents T_1 generation. N = number of flowers recorded (first five flower positions per plant); af=aberrant flowers; ↑=increase in organ number; ↓=decrease in organ number; s=sepals; p=petals; st=stamens; fst=forked stamen; pst=petaloid stamen; c=carpels; abd=arrested bud development. Data in percentages.



	FP1	FP2	FP3	FP4	FP5
Control	3	1	0	1	0
BAP-treated (T0)	79	55	61	61	50
T1	17	10	7	2	2
T2	14	1	1	4	0
T3	4	2	7	0	2

Figure 3.22. Occurrence of aberrant floral phenotypes in the first five flower positions (FP) of control, BAP-treated plants, and non-treated T_1, T_2, T_3 generations generated from BAP-treated plants. N= control 144; BAP-treated 217; T_1 41; T_2 36; T_3 56 plants, in at least three populations.

3.6.2 Inheritance of Floral Phenotypes in Low Temperatures

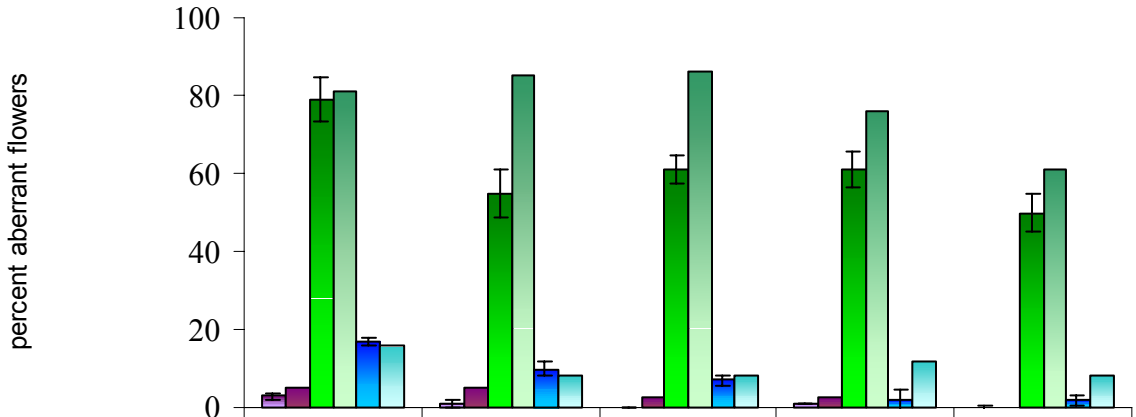
Unlike tomato (Sawhney, 1983), the floral phenotype of *Arabidopsis* was not affected by low temperatures alone. Plants treated with BAP and grown at low temperatures (18/13°C) showed similar phenotypes to treated plants grown at control temperatures (23/18°C), including aberrant floral phenotypes, increased lateral branching, and dark green leaves. Whereas 82% of BAP-treated plants produced at least one aberrant flower, at low temperatures it was 95-100%. T_1 offspring of BAP-treated plants grown at low temperatures showed similar aberrant floral phenotypes as BAP-treated progeny grown in control temperatures, but at a slightly higher rate of occurrence (Figure 3.23).

3.6.3 Inheritance of BAP-induced Transcript Levels

For generational comparison, the T_1 represented offspring of the BAP-treated R1 populations. The 247 genes identified by KDE® as having significant changes in transcript levels in the T_1 generation were categorized with GO (Figure 3.24). Categories found to be significant in the C2/T1 sample corresponded to ones affected in the BAP-treated replicates, including emphasis on cell wall, response to abiotic and biotic stimuli, and response to stress (categories defined in section 3.1.7.1).

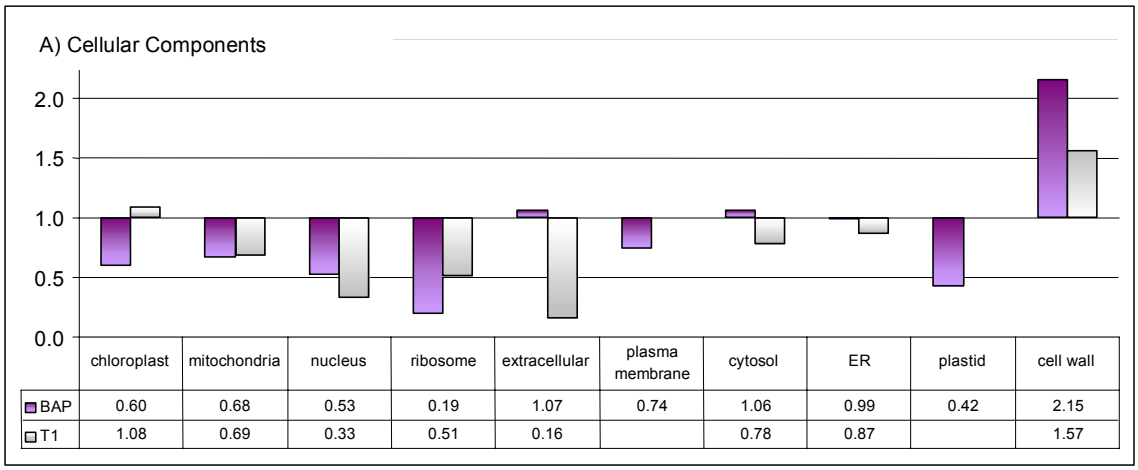
3.6.3.1 Light

Biological clocks are universal mechanisms for coordinating physiological processes synchronized to diurnal cycles of light and dark. Central components of biological clocks, *CCA1* and *LHY* (Wang et al., 1997; Schaffer et al., 1998) showed increased transcript levels in BAP-treated and T1 arrays (Table 3.34). *PCL1*, a MYB-like transcription factor with sequence similarity to type-B *ARRs* (Hazen et al., 2005), had lower transcript levels. *APRR1*, also known as *TOC1*, (Yamashino et al., 2003; Nakamichi et al., 2005) did not show significantly altered transcript levels in BAP-treated or T1 samples (Table 3.34). The 22k and 8k chip data conflicted as to whether BAP increased transcript abundance of *APPR9*. BAP treatment lowered transcript levels of *ELF4*, a gene associated with clock accuracy and flower timing (Doyle et al., 2002),

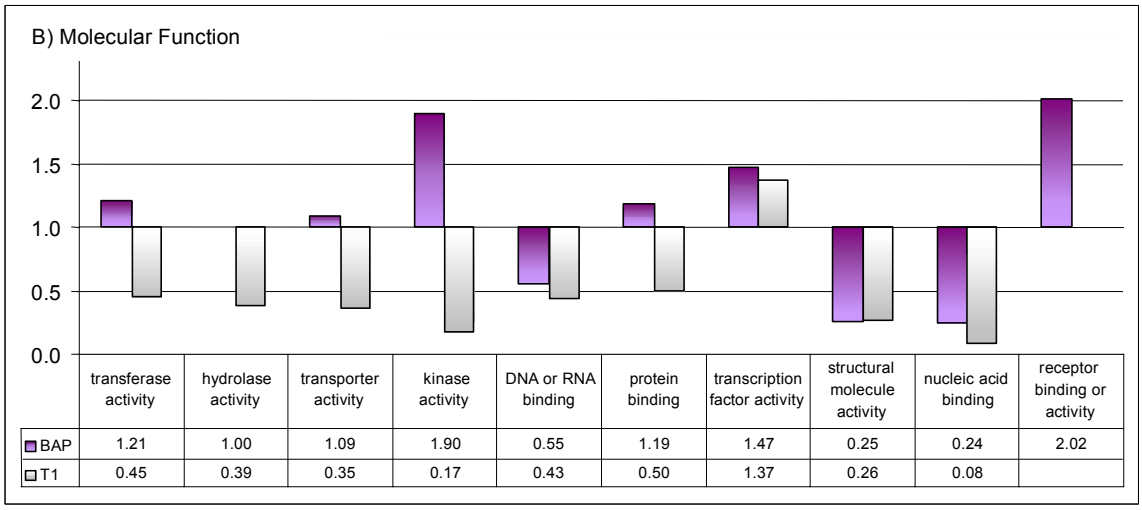


	FP 1	FP 2	FP 3	FP 4	FP 5
Control	3	1	0	1	0
Control + LTR	5	5	3	3	0
BAP (T0)	79	55	61	61	50
BAP + LTR (T0)	81	85	86	76	61
T1 (T0 BAP)	17	10	7	2	2
T1 (T0 BAP+ LTR)	16	8	8	12	8

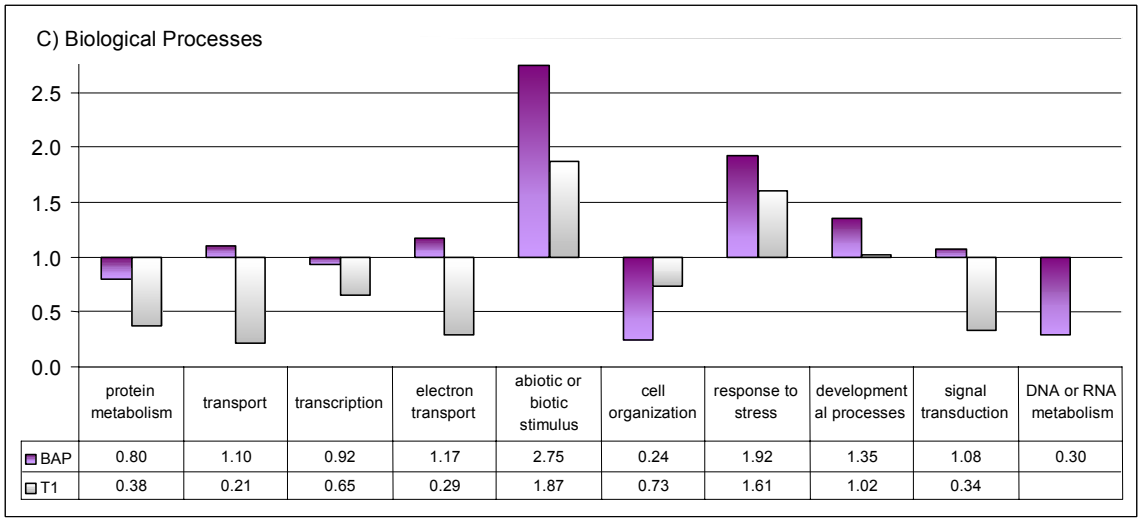
Figure 3.23. Occurrence of aberrant floral phenotypes in the first five flower positions of control, BAP-treated and non-treated T_1, T_2, T_3 generations. Plants grown under control (23/18°C) and low temperatures (18/13°C). Normal temperature: N= control 144 plants, BAP-treated 217 plants, and T_1(BAP) 41 plants, all in at least three populations. Low temperature: N=control+LTR, BAP+LTR, and T_1 BAP+LTR, 40 plants in each of two populations.



	chloroplast	mitochondria	nucleus	ribosome	extracellular	plasma membrane	cytosol	ER	plastid	cell wall
BAP	0.60	0.68	0.53	0.19	1.07	0.74	1.06	0.99	0.42	2.15
T1	1.08	0.69	0.33	0.51	0.16		0.78	0.87		1.57



	transferase activity	hydrolase activity	transporter activity	kinase activity	DNA or RNA binding	protein binding	transcription factor activity	structural molecule activity	nucleic acid binding	receptor binding or activity
BAP	1.21	1.00	1.09	1.90	0.55	1.19	1.47	0.25	0.24	2.02
T1	0.45	0.39	0.35	0.17	0.43	0.50	1.37	0.26	0.08	



	protein metabolism	transport	transcription	electron transport	abiotic or biotic stimulus	cell organization	response to stress	developmental processes	signal transduction	DNA or RNA metabolism
BAP	0.80	1.10	0.92	1.17	2.75	0.24	1.92	1.35	1.08	0.30
T1	0.38	0.21	0.65	0.29	1.87	0.73	1.61	1.02	0.34	

Figure 3.24. GO categorization of 653 significant genes from BAP-treated plants (purple) and 247 significant genes from the T_1 generation (silver), normalized to the frequency of all genes in the *Arabidopsis* genome.

Table 3.34. Microarray data of inherited transcript levels of genes associated with biological clock function.

	C1	R1	R2	R3	T1	C1Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	T1Signal T1:C1
Arabidopsis PSEUDO-RESPONSE REGULATOR 1 (APRR1) = TOC1 **At5g61380**										
22k	P	P	P	P	P	1762.01	1084.21	2425.19	1114.50	798.34
							-1.63	1.38	-1.58	**-2.21**
APRR9 **At2g46790**										
22k	A	P	A	P	A	68.68	185.46	66.48	293.45	17.91
							2.70	-1.03	**4.27**	**-3.84**
8k	A	P	A	P	P	70.61	195.51	59.69	216.39	125.03
							2.77	-1.18	**3.06**	1.77
CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) **At2g46830**										
22k	P	P	A	P	P	242.67	566.88	97.51	874.90	799.28
							2.34	**-2.49**	**3.61**	**3.29**
8k	A	P	A	P	P	86.22	232.58	25.79	454.99	280.60
							2.70	**-3.34**	**5.28**	**3.25**
LATE ELONGATED HYPOCOTYL (LHY) **At1g01060**										
22k	P	P	A	P	P	362.48	1296.69	38.41	3012.52	2652.32
							3.58	**-9.44**	**8.31**	**7.32**
8k	P	P	P	P	P	355.75	661.41	98.74	1496.61	1197.79
							1.86	**-3.60**	**4.21**	**3.37**
EARLY FLOWERING 4 (ELF4) **At2g40080**										
22k	P	P	P	A	A	323.56	116.38	506.02	49.01	56.55
							-2.78	1.56	**-6.60**	**-5.72**
8k	P	P	P	M	P	477.95	193.33	700.79	46.07	48.68
							-2.47	1.47	**-10.37**	**-9.82**
FLAVIN-BINDING KELCH DOMAIN F BOX PROTEIN 1 (FKF1) **At1g68050**										
22k	P	P	P	P	P	1058.43	518.08	1707.90	286.79	311.98
							-2.04	1.61	**-3.69**	**-3.39**
GIGANTEA (GI) **At1g22770**										
22k	P	P	P	P	P	1812.38	1060.93	2079.47	626.83	286.53
							-1.71	1.15	**-2.89**	**-6.33**
8k	P	P	P	P	P	2581.21	1626.62	3010.23	1409.39	540.20
							-1.59	1.17	-1.83	**-4.78**
PHYTOCHROME INTERACTING FACTOR 4 (PIF4) **At2g43010**										
22k	P	P	P	P	P	1191.97	519.58	852.47	320.07	421.32
							-2.29	-1.40	**-3.72**	**-2.83**
8k	P	P	P	P	P	1359.17	584.36	1103.14	237.78	363.63
							-2.33	-1.23	**-5.72**	**-3.74**
PHYTOCLOCK 1 (PCL1) **At3g46640**										
22k	P	P	P	P	P	1270.57	424.22	1036.41	289.82	381.73
							-3.00	-1.23	**-4.38**	**-3.33**

Ratios of transcript changes of genes associated with biological clocks in BAP-treated replicates (R1-3) and the next generation (C2/T1) compared to control (C1). Flags: P(resent); M(arginal); A(bsent). Red represents an increase and blue a decrease in transcript levels ≥ 2.5 fold, with lighter shade indicating a fold change < 2.5 and ≥ 2.

and this was carried to the next generation. BAP treatment did not alter transcript levels of *ZEITLUPE* (*ZTL*), a gene linked to PHYB regulation of circadian rhythms via protein degradation (Kevei et al., 2006).

BAP heritably lowered transcript levels of *FLAVIN-BINDING KELCH DOMAIN F BOX PROTEIN 1* (*FKF1*) and *GIGANTEA* (*GI*), genes serving in flower timing (Table 3.35). An important convergence point in photoperiodic induction of flowering (Imaizumi et al., 2005), *CO* was flagged A(bsent).

Additional genes known to have transcript levels entrained in an oscillating diurnal period (Harmer et al., 2000; Davis and Millar, 2001) were mined from the data (Table 3.35). For example, *SEN1* transcript has been described as differentially regulated at the level of mRNA stability in a circadian manner (Schenk et al., 2005).

BAP also lowered transcript levels, in both the treated and T_1 generation, of *PIF4*, a gene that negatively regulates PHYB responses (Huq and Quail, 2002). An unknown gene, At4g04330, also showed decreased transcript levels in treated and T_1 plants. BAP treatment increased transcript levels of *DIN11*, a gene upregulated by sugar-stress in senescent leaves and by a lack of light (Fujiki et al., 2001). Functioning in chloroplast development, *PORA*, *PORB*, and *PORC* showed lower transcript abundance in both the BAP-treated and T_1 generations (Table 3.36).

Many components of photosynthetic processes are entrained in circadian rhythms, with metabolic oscillations likely having evolved to maximize the efficiency of the light harvesting processes. In response to light stimuli perceived by phytochromes, and within the context of circadian clock entrainment, chloroplast membranes acquire proteins of the CHLOROPHYLL A/B BINDING PROTEIN (CAB)-family, for example, in tobacco and pea, *CAB* transcript is minimal at night and increases 2-4 h before dawn (Kloppstech, 1985; Nagy et al., 1986). Unfortunately *CAB1&2* genes were not represented on the microarrays used in the present study. *ELIP1,2*, light-induced members of the CAB family encoding thylakoid membrane proteins, were increased in both BAP-treated and T_1 generation plants (Table 3.36). Some genes associated with anthocyanin production had increased transcript abundance in BAP-treated samples, and for most this increase appeared to be heritable (Table 3.37).

Table 3.35. Microarray data of inherited transcript levels of genes known to be entrained by circadian clocks or showing the pattern proposed to reflect circadian rhythms.

	C1	R1	R2	R3	T1	C1Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	T1Signal T1:C1
***CONSTANS-LIKE* with photoperiod sensitivity At1g07050**										
22k	P	P	P	A	P	670.77	236.51	814.80	78.66	102.74
							-2.84	1.21	**-8.53**	**-6.53**
***CONSTANS-LIKE* B-box zinc finger protein At2g21320**										
22k	A	P	A	P	P	102.26	585.65	179.51	552.41	499.55
							5.73	1.76	**5.40**	**4.89**
8k	A	P	M	P	P	43.45	476.07	126.75	506.82	410.40
							10.96	2.92	**11.66**	**9.45**
***DARK INDUCIBLE 11* (*DIN11*) At3g49620**										
22k	M	P	A	P	P	62.57	768.86	73.87	806.53	816.24
							12.29	1.18	**12.89**	**13.04**
***SENESCENCE-ASSOCIATED 1* (*SEN1*) At4g35770**										
22k	P	P	P	P	P	3375.98	1729.92	3124.01	880.34	1158.39
							-1.95	-1.08	**-3.83**	**-2.91**
8k	P	P	P	P	P	1363.93	855.47	1292.52	376.00	394.17
							-1.59	-1.06	**-3.63**	**-3.46**
***STO HOMOLOG* (*STH*) At2g31380**										
22k	P	P	P	P	P	492.20	1027.89	276.28	1253.66	1298.83
							2.09	-1.78	**2.55**	**2.64**
8k	P	P	P	P	P	475.24	965.94	134.12	1157.63	1591.01
							2.03	**-3.54**	**2.44**	**3.35**
transcription controlled by circadian clock At3g26740										
22k	P	P	P	P	P	12920.92	5485.60	11954.56	2277.39	1228.14
							-2.36	-1.08	**-5.67**	**-10.52**
8k	P	P	P	P	P	13106.32	6055.13	13664.30	2926.57	1480.30
							-2.16	1.04	**-4.48**	**-8.85**
unknown light responsive At4g04330										
22k	P	P	P	A	P	1934.47	315.35	1195.23	35.70	147.04
							-6.13	-1.62	**-54.19**	**-13.16**
8k	P	P	P	A	P	1555.38	398.30	1112.72	26.33	186.11
							-3.91	-1.40	**-59.08**	**-8.36**

Ratios of transcript changes of genes associated with biological clocks in BAP-treated replicates (R1-3) and the next generation (C2/T1) compared to control (C1). Flags: P(resent); M(arginal); A(bsent). Red represents an increase and blue a decrease in transcript levels ≥ 2.5-fold, with lighter shades indicating a fold change < 2.5 and ≥ 2.

Table 3.36. Microarray data of inherited transcript levels of genes associated with light responses

	C1	R1	R2	R3	T1	C1Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	T1Signal T1:C1
PROTOCHLOROPHYLLIDE OXIDOREDUCTASE A (PORA) **At5g54190**										
22k	P	P	P	A	P	1158.40	223.00	369.36	217.82	507.09
							-5.19	**-3.14**	**-5.32**	**-2.28**
8k	P	A	A	A	P	573.68	8.72	8.84	9.05	226.20
							-65.77	**-64.88**	**-63.39**	**-2.54**
PORB **At4g27440**										
22k	P	P	P	P	P	21502.81	8285.47	13539.09	2299.18	9968.36
							-2.60	-1.59	**-9.35**	**-2.16**
8k	P	P	P	P	P	7223.58	2665.97	3969.67	725.68	2027.18
							-2.71	-1.82	**-9.95**	**-3.56**
PORC **At1g03630**										
22k	P	P	P	P	P	5497.42	3299.17	2643.11	1639.68	3561.88
							-1.67	**-2.08**	**-3.35**	-1.54
8k	P	P	P	P	P	2124.30	1464.54	954.28	828.52	1402.04
							-1.45	**-2.23**	**-2.56**	-1.52
EARLY LIGHT-INDUCED PROTEIN 1 (ELIP1) **At3g22840**										
22k	P	P	P	P	P	262.51	3555.96	137.40	1373.45	2121.67
							13.55	-1.91	**5.23**	**8.08**
ELIP2 **At4g14690**										
22k	A	P	A	M	P	196.88	782.37	127.80	279.53	629.62
							3.97	-1.54	1.42	**3.20**

Ratios of transcript changes of genes associated with biological clocks in BAP-treated replicates (R1-3) and the next generation (C2/T1) compared to control (C1). Flags: P(resent); M(arginal); A(bsent). Red represents an increase and blue a decrease in transcript levels ≥ 2.5-fold, with lighter shade indicating a fold change < 2.5 and ≥ 2.

Table 3.37. Microarray data of inherited transcript levels of genes associated with flavonoid biosynthesis

	C1	R1	R2	R3	T1	C1Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	T1Signal T1:C1
CHALCONE SYNTHASE (CHS) (= TT4) **At5g13930**										
22k	P	P	P	P	P	3750.67	14434.81	3544.33	6847.91	10211.53
							3.85	-1.06	1.83	**2.72**
8k	P	P	P	P	P	3161.68	11737.41	2738.31	6202.82	9922.10
							3.71	-1.15	1.96	**3.14**
PHENYLALANINE AMMONIA-LYASE 1 (PAL1) **At2g37040**										
22k	P	P	P	P	P	2158.06	5262.60	2441.44	5465.99	3865.38
							2.44	1.13	**2.53**	1.79
8k	P	P	P	P	P	978.31	2250.96	1378.74	2867.33	1445.94
							2.30	1.41	**2.93**	1.48
TRANSPARENT TESTA 3 (TT3) **At5g42800**										
22k	P	P	P	P	P	621.17	5372.23	583.58	3455.42	1646.63
							8.65	-1.06	**5.56**	**2.65**
8k	P	P	P	P	P	148.68	797.32	101.69	583.34	165.11
							5.36	-1.46	**3.92**	1.11
TT5 **(= *CHALCONE ISOMERASE (CHI)*) At5g13930**										
22k	P	P	P	P	P	647.11	1841.80	679.61	1196.78	1596.67
							2.85	1.05	1.85	**2.47**
TT6 **At3g51240**										
22k	P	P	P	P	P	1515.53	5695.09	559.94	3892.87	3632.57
							3.76	**-2.71**	**2.57**	**2.40**
8k	P	P	P	P	P	944.36	4030.21	440.67	3150.36	2975.87
							4.27	**-2.14**	**3.34**	**3.15**
TT7 **At5g07990**										
22k	A	P	M	P	P	147.28	963.32	173.60	831.94	238.46
							6.54	1.18	**5.65**	1.62
TT19 **At5g17220**										
22k	P	P	P	P	P	195.36	1614.30	202.41	1037.05	641.87
							8.26	1.04	**5.31**	**3.29**
chalcone-flavanone isomerase family At5g05270										
22k	A	P	A	P	P	295.32	905.51	169.90	656.48	738.96
							3.07	-1.74	**2.22**	**2.50**

Ratios of transcript changes of genes associated with biological clocks in BAP-treated replicates (R1-3) and the next generation (C2/T1) compared to control (C1). Flags: P(resent); M(arginal); A(bsent). Red represents an increase and blue a decrease in transcript levels ≥ 2.5-fold, with lighter shades indicating a fold change < 2.5 and ≥ 2.

3.6.3.2 Low Temperature

KDE® identified that several cold responsive genes had significantly lower transcript levels in BAP-treated populations and the next generation. Transcript levels of *CBF1* and *COR15b* were 5-fold lower in the BAP-treated populations and over 7-fold in the next generation (Table 3.38). Expression of *INDUCER OF CBF EXPRESSION 1* (*ICE1*), a cold acclimation factor functioning upstream of *CBF1* (Chinnusamy et al., 2003), was not affected in the BAP-treated populations. Meta Analyzer data showed that zeatin slightly decreases levels of *CBF1* and *COR15a* and ethylene decreases *CBF1, CBF2, CBF3, COR15a, COR15b, COR47, COR78, COR 413, COR414,* and *KIN1*. The same database showed transcript levels of *COR* genes are induced by cold and that light also increases transcript abundance, with the exception of *COR15a*, which responds to light treatments with lowered transcript levels (Figure 3.25).

Proline functions as a cellular osmolyte under stress conditions, such as cold, stabilizing proteins, membranes, and subcellular structures and protecting cells from degradation by scavenging reactive oxygen species (Bohnert and Shen, 1999). The microarray data here indicated BAP treatment decreased transcript levels of *PYROLINE-5-CARBOXYLATE SYNTHETASE 1* (*P5CS1*) in a heritable manner (Table 3.38). Also functioning in cold response, *RARE-COLD-INDUCIBLE 2B* (*RCI2B*) had significantly reduced transcript levels in BAP samples, but this expression pattern was not carried into the T1.

3.6.3.3 Defense

The C2/T1 data was mined for evidence of heritable defense-responses. Transcript levels of *PLANT DEFENSIN PROTEIN 1.2* (*PDF1.2*) and *PDF-LIKE*, encoding proteins with antifungal activity and defense activity, had decreased transcript levels in BAP-treated populations, but increased levels in the next generation (Table 3.39). *POLYGALACTURONASE INHIBITING PROTEIN 1* (*PGIP1*), encoding a LRR protein, and a member of the carboxyl methyltransferase family (At1g66690), with a role in the defense response, had significantly increased transcript abundance in BAP-treated samples and the T_1 generation. Two unknown function genes, At4g16146 and

Table 3.38. Microarray data of inherited transcript levels of genes associated with cold acclimation

	C1	R1	R2	R3	T1	C1Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	T1Signal T1:C1
C-REPEAT/DRE BINDING FACTOR 1 (CBF1) **At4g25490**										
22k	A	A	A	A	A	61.05	10.51	27.33	4.84	7.54
							-5.81	**-2.23**	**-12.61**	**-8.10**
8k	A	A	P	A	A	167.69	47.97	84.01	60.88	33.40
							-3.50	**-2.00**	**-2.75**	**-5.02**
CBF2 **At4g25470**										
22k	A	A	M	A	A	185.43	83.34	115.24	71.40	63.15
							-2.22	-1.61	**-2.60**	**-2.94**
8k	P	P	P	P	P	723.04	490.60	583.62	533.97	411.35
							-1.47	-1.24	-1.35	-1.76
CBF3 **At4g25480**										
22k	P	A	P	A	A	117.52	65.32	90.86	49.01	64.09
							-1.80	-1.29	**-2.40**	-1.83
8k	P	P	P	P	P	241.01	196.24	221.81	220.50	201.38
							-1.23	-1.09	-1.09	-1.20
COLD-RESPONSIVE PROTEIN 15a (COR15a) **At2g42540**										
22k	P	P	P	A	P	312.87	160.68	148.48	62.92	127.24
							-1.95	**-2.11**	**-4.97**	**-2.46**
COR15b **At2g42530**										
22k	P	P	P	P	P	1696.39	340.13	863.55	171.23	228.10
							-4.99	-1.96	**-9.91**	**-7.44**
8k	P	P	P	M	P	1905.02	250.03	950.60	78.16	161.30
							-7.62	**-2.00**	**-24.37**	**-11.81**
COR47 **At1g20440**										
22k	P	P	P	P	P	6886.27	2165.41	4461.07	2368.76	4790.01
							-3.18	-1.54	**-2.91**	-1.44
8k	P	P	P	P	P	3457.00	1354.06	2456.82	1551.73	2492.93
							-2.55	-1.41	**-2.23**	-1.39
COR78 **At5g52310**										
22k	P	P	P	P	P	2889.12	1255.40	1508.45	592.34	1108.43
							-2.30	-1.92	**-4.88**	**-2.61**
8k	P	P	P	P	P	2272.31	597.45	1048.61	441.82	987.82
							-3.80	**-2.17**	**-5.14**	**-2.30**
COR413 **At2g15970**										
22k	P	P	P	P	P	4808.33	1861.32	2988.82	995.30	2543.93
							-2.58	-1.61	**-4.83**	-1.89
8k	P	P	P	P	P	4405.44	2378.88	3163.50	986.49	2543.52
							-1.85	-1.39	**-4.47**	-1.73
COR414 **At1g29395**										
22k	P	M	P	A	A	1062.24	370.91	468.34	284.37	316.70
							-2.86	**-2.27**	**-3.74**	**-3.35**

***COLD INDUCIBLE 1* (*KIN1*) At5g15960**										
22k	P	P	P	P	P	11273.37	4643.92	5209.39	2303.41	4540.24
							-2.43	**-2.16**	**-4.89**	**-2.48**
8k	P	P	P	P	P	13118.54	5296.33	6330.69	2790.82	5247.38
							-2.48	**-2.07**	**-4.70**	**-2.50**
***KIN2* At5g15970**										
8k	P	P	P	P	P	11576.74	4818.09	6486.18	3271.31	4659.46
							-2.40	-1.78	**-3.54**	**-2.48**
***INDUCER OF CBF EXPRESSION 1* (*ICE1*) At3g26744**										
22k	P	P	P	P	P	2941.78	2996.58	2619.47	2414.13	3182.97
							1.02	-1.12	-1.22	1.08
***PYRROLINE-5-CARBOXYLATE SYNTHASE 1* (*P5SC1*) At2g39800**										
8k	P	P	P	P	P	2171.83	625.06	699.32	572.64	629.91
							-3.47	**-3.11**	**-3.79**	**-3.45**
***RARE-COLD-INDUCIBLE 2B* (*RCI2B*) At3g05890**										
22k	P	P	P	P	P	1095.06	204.23	402.60	265.01	1070.73
							-5.36	**-2.72**	**-4.13**	-1.02

Ratios of transcript changes of genes associated with biological clocks in BAP-treated replicates (R1-3) and the next generation (C2/T1) compared to control (C1). Flags: P(resent); M(arginal); A(bsent). Blue represents a decrease in transcript levels ≥ 2.5-fold, with lighter shade indicating a fold change < 2.5 and ≥ 2.

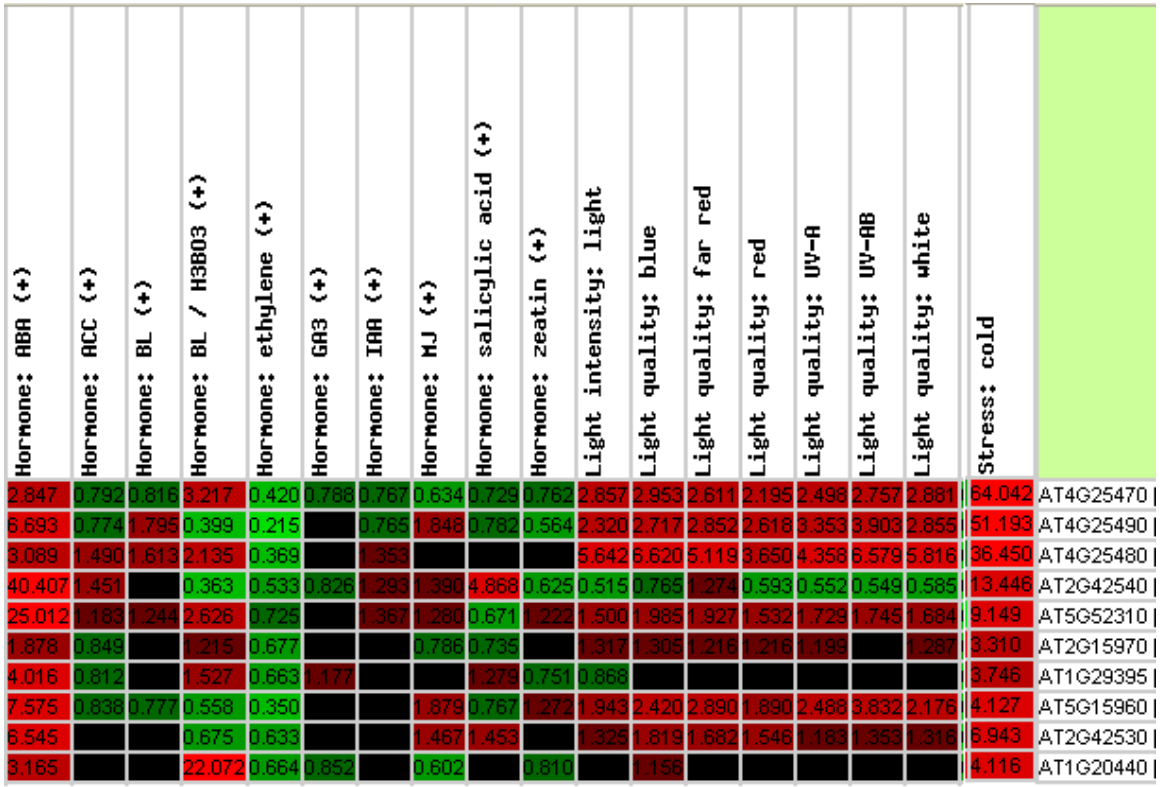


Figure 3.25. Meta Analyzer data for response of *COR* genes to hormone, light, and cold treatments. Red indicates increased transcript levels and green decreased.

Table 3.39. Microarray data of inherited transcript levels of genes associated with stress and defense responses

	C1	R1	R2	R3	T1	C1Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	T1Signal T1:C1
AIG1-like **At2g33830**										
22k	P	P	P	P	P	22183.50	6039.72	15169.43	766.59	2956.76
							-3.67	-1.46	**-28.94**	**-7.50**
8k	P	P	P	P	P	20408.66	4667.63	14431.41	964.28	2695.27
							-4.37	-1.41	**-21.16**	**-7.57**
PLANT DEFENSIN PROTEIN 1.2 (PDF1.2) **At5g44420**										
22k	P	P	A	M	P	147.28	94.61	22.16	58.69	840.75
							-1.56	**-6.65**	**-2.51**	**5.71**
PDF-like **At2g26020**										
22k	P	P	A	P	P	202.99	407.70	62.05	260.17	3900.25
							2.01	**-3.27**	1.28	**19.21**
POLYGALACTURONASE INHIBITING PROTEIN 1 (PGIP1) **At5g06860**										
22k	P	P	P	P	P	248.01	708.04	831.79	1058.83	748.38
							2.85	**3.35**	**4.27**	**3.02**
cell wall modification At2g45220										
22k	A	P	P	P	M	60.29	1863.57	610.18	4287.36	305.38
							30.91	**10.12**	**71.12**	**5.07**
8k	P	P	P	P	P	179.91	1244.31	381.71	2525.89	191.84
							6.92	**2.12**	**14.04**	1.07
defense response carboxyl methyltransferase family At1g66690										
22k	P	P	P	P	P	385.37	1622.55	593.18	9397.58	1985.00
							4.21	1.54	**24.39**	**5.15**
heat shock protein hsp20 family At1g06460										
22k	P	P	P	P	P	2201.56	834.93	1741.88	193.61	347.80
							-2.64	-1.26	**-11.37**	**-6.33**
vicinity of R-haplotype At4g16146										
22k	P	P	P	P	P	978.30	396.44	1269.84	333.38	265.80
							-2.47	1.30	**-2.93**	**-3.68**
vicinity of R-haplotype At4g16515										
22k	P	P	P	P	P	1491.11	358.90	1857.86	464.07	411.89
							-4.15	1.25	**-3.21**	**-3.62**

Ratios of transcript changes of genes associated with defense in BAP-treated replicates (R1-3) and the next generation (C2/T1) compared to control (C1). Flags: P(resent); M(arginal); A(bsent). Red represents an increase and blue a decrease in transcript levels ≥ 2.5-fold, with lighter shades indicating a fold change < 2.5 and ≥ 2.

At4g16515, located in the vicinity of the R-haplotype on chromosome IV (The EU Arabidopsis Genome Project et al., 1998), showed decreased transcript abundance in the BAP-treated and T_1 populations. *SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1* (*SNC1*), (At4g16890), of the R-haplotype, was flagged A(bsent) on all of the arrays.

3.6.3.4 Miscellaneous

Two auxin-responsive genes, *ARF12* and *IAA2*, appeared to respond to BAP in a heritable manner (Table 3.40). A heritable increase in transcript levels was found for *LIPID TRANSFER PROTEIN 2* (*LTP2*), a gene reported to be expressed in floral meristems, flowers, and seeds (Clark and Bohnert, 1999). *LTP1*, *LTP6*, *LTP12*, *LTP3*, and *LTP5* maintained steady state transcript levels in BAP-treated samples and the next generation. *LTP4* responded to BAP treatment, while adjacent locus *LTP3* did not; both genes have been reported as upregulated by ABA (Arondel et al., 2000). *NITRATE TRANSPORTER 2:1* (*NRT2:1*) had heritable lowered transcript abundance in BAP-treated populations. Meta Analyzer indicates *NRT2:1* is also repressed by zeatin. BAP induced a heritable decrease in transcript levels of a gene encoding a membrane protein, At4g17340. Three ribosomal proteins displayed increased transcript levels in the next generation, although none were affected in the BAP-treated populations. The pattern of response for the heat shock protein At1g06460, two genes associated with dormancy At2g33830 and At1g28330, and *LTP4* resembled that of genes with expression entrained by the circadian clock.

3.6.3.5 Promoter Motifs

The 247 genes identified by KDE® as having significantly altered transcript levels in the T_1 population were analysed for known cis-elements in the upstream 1000 bp regulatory region. The evening element 5'-AAATATCT-3', associated with circadian clock function, appeared 80 times in the upstream sequences of the 247 genes. The methylation-friendly G-box, 5'-CACGTG-3', present in many genes mediating responses to light, elicitors, and redox changes (Staiger et al., 1989), was found 45 times in the significant T_1 genes.

Table 3.40. Microarray data of inherited transcript levels of miscellaneous genes

	C1	R1	R2	R3	T1	C1Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	T1Signal T1:C1
AUXIN RESPONSE FACTOR 12 (ARF12) At1g34310										
22k	P	P	P	P	P	3836.90	910.01	2406.72	842.22	1552.37
							-4.22	-1.59	**-4.56**	**-2.47**
8k	A	A	A	A	A	16.29	2.18	3.68	0.82	4.77
							-7.47	**-4.42**	**-19.80**	**-3.41**
DORMANCY-ASSOCIATED PROTEIN 1 (DRM1) At1g28330										
22k	P	P	P	P	P	3871.24	1078.20	3703.16	450.15	457.13
							-3.59	-1.05	**-8.60**	**-8.47**
8k	P	P	P	P	P	14118.58	5298.51	14384.25	2224.75	3195.38
							-2.66	1.02	**-6.35**	**-4.42**
INDOLEACETIC ACID-INDUCED PROTEIN 2 (IAA2) At3g23030										
22k	P	P	P	P	P	1919.21	933.29	1086.64	1301.45	491.07
							-2.06	-1.77	-1.47	**-3.91**
8k	P	P	P	P	P	507.82	230.40	507.72	255.88	122.17
							-2.20	-1.00	-1.98	**-4.16**
LIPID TRANSFER PROTEIN 2 (LPT2) At2g38530										
22k	P	P	P	P	P	1288.12	3542.44	5364.52	4793.78	2918.12
							2.75	**4.16**	**3.72**	**2.27**
8k	P	P	P	P	P	1448.79	2001.66	6584.92	4078.44	4926.69
							1.38	**4.55**	**2.82**	**3.40**
***LTP4* At5g59310**										
22k	P	P	P	P	P	3307.30	275.56	1764.04	206.93	935.00
							-12.00	-1.87	**-15.98**	**-3.54**
NITRATE TRANSPORTER 1 (NRT2:1) At1g08090										
22k	P	A	P	P	A	1335.44	145.66	236.39	499.16	193.22
							-9.17	**-5.65**	**-2.68**	**-6.91**
8k	P	M	P	P	A	1198.27	170.80	383.92	476.38	105.94
							-7.02	**-3.12**	**-2.52**	**-11.31**
membrane protein At4g17340										
22k	P	P	P	P	P	3950.60	1696.08	465.89	1048.11	364.16
							-2.33	**-8.48**	**-3.77**	**-10.85**
8k	P	P	P	P	P	2658.60	1851.82	595.68	1449.76	417.19
							-1.44	**-4.46**	-1.83	**-6.37**
ribosomal protein L19B At3g16780										
22k	P	P	P	P	P	2470.94	3390.77	3424.66	3649.64	6873.04
							1.37	1.39	1.48	**2.78**
ribosomal protein S25E At4g39200										
22k	P	P	P	P	P	6304.78	10554.49	8082.97	8987.36	18196.77
							1.67	1.28	1.43	**2.89**
ribosomal protein S8B At5g59240										
22k	M	P	P	P	P	190.01	283.06	258.55	280.14	531.60
							1.49	1.36	1.47	**2.80**

Ratios of transcript changes of genes in BAP-treated replicates (R1-3) and the next generation (C2/T1) compared to control (C1). Flags: P(resent); M(arginal); A(bsent). Red represents an increase and blue a decrease in transcript levels ≥ 2.5-fold, with lighter shades indicating a fold change < 2.5 and ≥ 2.

3.6.4 Isolating T_1 Plants

Further molecular analysis of T_1 genes linked to heritable floral phenotypes was restricted by the inability to determine plants with epigenetically altered gene expression prior to flower development. Early identification of T_1 plants carrying heritable BAP-induced expression patterns was attempted based on elongated hypocotyls, a phenotype associated with high transcript levels of *LHY* or *CCA1* (Schaffer et al., 1998; Wang and Tobin, 1998); however, hypocotyl lengths were consistent between controls and the six T_1 populations that were tested.

3.6.5 Factors Affecting Transcriptional Competency

Of the 247 significant genes identified for the C2/T1 array, 72% showed a decrease in transcript levels; therefore, a mechanism invoking gene silencing was suspected in BAP-induced epigenetic inheritance and evidence of such was sought in the data. Heritable gene silencing can involve de novo methylation of DNA cytosine bases by *CHROMOMETHYLASE 3* (*CMT3*) or *MET1* (Jackson et al., 2002a). Both of these genes maintained steady state transcript levels in BAP-treated populations (Table 3.41). Chromatin remodelling by histone acetylation is another method of altering gene expression patterns in developmental cell lineages and between generations. Acetylation and deacetylation of histones depends on the action of highly conserved enzymes, respectively HISTONE ACETYLTRANSFERASES (HAT) and HISTONE DEACETYLTRANSFERASES (HDAC) (Pandey et al., 2002). Genes associated with alteration of histone chemistry were mined from the data, but, again, did not show significant change in transcript levels in BAP-treated populations (Table 3.41). Functioning in chromatin remodelling in the shoot meristem, *FAS1* and *FAS2*, maintained steady state transcript levels (Table 3.41). *TSK*, a gene associated with the cell cycle and gene silencing (Takeda et al., 2004), was not represented on the microarrays. Relatively recently, the action of small RNA molecules (miRNA) has been linked to epigenetic inheritance. By regulating miRNA, *DCL1* in *Arabidopsis* has the capacity to mediate gene expression (Schauer et al., 2002).

Table 3.41. Microarray data of genes related to chromatin-remodelling and potential mechanism of epigenetic inheritance

	C1	C2	R1	R2	R3	C1Signal	C2Signal	R1Signal R1:C1	R2Signal R2:C1	R3Signal R3:C1	SAM	Gene-Spring
***CHROMOMETHYLASE 3* (*CMT3*) At1g69770**												
22k	P	P	P	P	P	831.02	1176.30	1015.13	941.12	720.61		
								1.22	1.13	-1.15	--	--
***DICER-LIKE 1* (*DCL1*) At1g01040**												
22k	A	A	P	A	P	422.00	405.29	554.12	545.17	736.34		
								1.31	1.29	1.74	--	--
***FASCIATA 1* (*FAS1*) At1g65470**												
22k	P	P	P	P	P	254.11	274.28	217.74	245.99	228.10		
								-1.17	-1.03	-1.11	--	--
***FAS2* At5g64630**												
22k	M	A	P	A	P	593.70	589.09	569.88	741.67	534.26		
								-1.04	1.25	-1.11	--	--
***HISTONE ACETYLTRANSFERASE 1* (*HAT1*) At3g54610**												
22k	A	A	A	M	A	336.53	352.51	312.35	322.82	421.11		
								-1.08	-1.04	1.25	--	--
***HISTONE DEACETYLASE 5* (*HDA5*) At5g61060**												
22k	P	P	P	P	P	410.55	348.74	827.42	819.23	1064.28		
								2.02	2.00	2.59	--	--
***HDA19* At4g38130**												
22k	P	P	P	P	P	1927.61	2416.69	1734.43	2237.55	2111.01		
								-1.11	1.16	1.10	--	--
***MERISTEM PROTEIN 5B* (*MERI5B*) At4g30270**												
22k	P	P	P	P	P	8789.46	6725.06	6307.02	7328.75	11074.77		
								-1.39	-1.20	1.26	--	--
8k	P	P	P	P	P	10529.87	7184.84	7888.17	8236.31	12211.46		
								-1.33	-1.28	1.16	--	
***MET1* At5g49160**												
22k	P	P	P	P	P	953.88	1156.50	1042.91	957.37	720.00		
								1.09	1.00	-1.32	--	--
8k	P	P	P	P	P	473.88	613.69	567.65	457.61	663.97		
								1.20	-1.04	1.40	--	
***MULTICOPY SUPPRESSOR OF IRA 1* (*MSI1*) At5g58230**												
22k	P	P	P	P	P	2028.34	2036.84	2053.53	2143.74	2516.39		
								1.01	1.06	1.24	--	--
8k	P	P	P	P	P	2418.27	3003.55	2755.37	2785.47	3495.92		
								1.14	1.15	1.45	--	
linked to S-adenosylmethionine-dependent methyltransferase activity At4g22530												
22k	A	P	P	P	P	156.44	149.86	415.96	503.06	620.17		
								2.66	3.22	3.96	***	***
linked to S-adenosylmethionine-dependent methyltransferase activity At3g54150												
22k	P	P	P	P	P	136.60	120.65	563.13	481.64	1015.87		
								4.12	3.53	7.44	**	**

Flags: P(resent); M(arginal); A(bsent). C1-2=controls, R1-3=BAP-treated replicates. SAM® and GeneSpring® statistical analysis: *** top, **middle, *bottom of significance range (Table 3.2); -- not recognized as significant; red is increased transcript levels.

4. DISCUSSION

The ambitious task of understanding cytokinin regulation of plant development and responses to environmental conditions can be tackled at the transcript level, with the results serving as early indications of subsequent functional responses. The challenge of presenting interpretations of the transcriptomic response to a master hormone will inevitably range from broad categories to fragmented observations. Data regarding this range were presented here, to provide an overview that supports and expands our present understanding and to recognize potentially novel aspects of cytokinin regulation of plant processes. In some cases, coincidental observations of cytokinin-influenced morphological phenotypes provide context and consequence to cytokinin-induced changes in transcript abundance. Finally, although the interpretation and mechanisms are speculative, intriguing observations of the inheritance of BAP-altered phenotypes and transcript levels are discussed. For the sake of effective comparison, specific examples of epigenetic inheritance are dispersed throughout the discussion, to facilitate comparisons with corresponding BAP-induced alterations in the treated samples.

4.1 Cytokinins, Shoot Meristems, and Flower Development

The present study investigated potential links between BAP-induced altered floral phenotypes and changes to the transcriptome to determine the role of cytokinins in the regulation of flower development. Aberrant floral phenotypes were also documented in subsequent generations; however, the ephemeral nature of induced floral phenotypes within the raceme and between generations suggested that any chromatin remodelling invoked by BAP treatment had the capacity to revert to pre-treatment states relatively rapidly, inhibiting detailed analysis of BAP-induced epigenetic effect. Floral phenotype is responsible for promoting the fertilization and maturation of ovules/seeds, and as such it would be expected that evolution would likely select for the canalization and recovery of viable floral morphology.

4.1.1 The Shoot Meristem and Increased Organ Number Phenotype

In the present study, BAP treatment was shown to decrease transcript levels of *CLV1* over 96 h (Table 3.6, Figure 3.12), coupled with increased meristematic size or activity; the latter was indicated by an increase in floral organ number and robust rachis diameters (Figures 3.7, 3.8). Interpretation of this phenotypic response as cytokinin-specific is supported by increased peduncle diameters in *HS:ipt* transgenic *Arabidopsis* (Rupp et al., 1999).

While predominately characterized as a meristematic gene, the present study showed that *CLV1* is expressed in all tissues of *Arabidopsis* (Figure 3.15). This suggests that BAP-induced changes in transcript abundance in the shoot meristem may have been partially masked by potential expression changes in other organs. Harvesting young, small plants and removing leaves minimized this possibility. However, root tissues were included and BA-treated *ahk4* mutant (*AHK4* serves in two-component systems of cytokinin signalling) show a significant decrease in *CLV1* transcript levels in roots (Rashotte et al., 2003). Therefore, in the present study, detection of BAP-lowered *CLV* transcript levels may have been reflecting changes in both shoot and root meristems. Although the role of *CLV1* in root development is not known, the short, dense rooting observed in BAP-treated plants may be connected to reduced *CLV1* expression.

The upstream region of *CLV1* includes the G-box promoter motif, 5'-CACGTG-3', a regulatory sequence associated with UV-B light induction of anthocyanin accumulation and hypocotyl elongation (Ulm et al., 2004). The presence of this motif suggests potential convergent regulation of shoot development and function by light-stimuli and cytokinins. This motif also provides a potential mechanism for BAP-induced epigenetic inheritance by chromatin remodelling, as the CpNpG base-pattern of the G-box is predisposed to cytosine methylation (Staiger et al., 1989).

That control transcript levels of *WUS* were not detected at 4 and 24 h by RT-PCR (Figure 3.12) probably reflected the naturally low transcript abundance of this transcription factor, which is normally restricted to a small region beneath the outermost three layers of the inflorescence meristem (Schoof et al., 2000). By 48 h (equivalent to the microarray sample collection) more floral meristems would have been initiated, coincident with detectable levels of *WUS* in control plants. Compared to controls, BAP

treatment elevated *WUS* transcript levels over 96 h. The high number of PCR cycles required for DNA amplification in all samples indicated relatively low levels of *WUS*, explaining the A(bsent) flags in control and BAP-treated microarrays.

Control levels of *WUS* transcript at 192 h were substantially higher than those at earlier time points (Figure 3.12), consistent with the peak in *WUS* transcript, indicated by a microarray database, approximately 8 days after the 4-5 rosette leaf stage (Figure 3.14). BAP-treated plants did not flower before controls; so accelerated development was not responsible for the difference in expression levels. At the time of BAP treatment, the shoot apex had recently transformed from a vegetative to an inflorescence meristem, bearing one to three floral buds. These proximal flowers of the raceme had not yet produced stamen and carpel primordia. The control and treated plants for the 192 h time point were harvested when the most proximal flowers were approximately at stage 10 (Smyth et al., 1990) and the corresponding spike in *WUS* transcript likely reflected increased expression in developing ovules and anthers (Wellmer et al., 2004).

The BAP-induced increase in the abundance of *WUS* transcript during the flowering time course in the present study was interpreted as integral to the coincident increased floral organ number phenotype (Figure 3.13). *Arabidopsis* transgenics, with *WUS* under the control of a *CLV1*, *AP3*, or *LFY* promoter, showed extra floral organ production, suggesting that increased and/or ectopic *WUS* expression is sufficient to cause an increased floral organ phenotype, as found in *clv1* mutants (Schoof et al., 2000; Lohmann et al., 2001). The capacity to influence transcript levels of the meristematic genes *CLV* and/or *WUS* may serve as an important mechanism in cytokinin regulation of shoot development.

The present study also included analysis of *WUS* and *CLV1* transcript abundance in *ampl*, an *Arabidopsis* mutant with increased cytokinin levels and extra organ floral phenotype (Chaudhury et al., 1993). An increase in transcript abundance of *WUS* seemed to occur independent of the *CLV1* pathway, warranting further investigation to determine the source of increased *WUS* transcript levels in *amp1*.

Two-component systems of cytokinin signal transduction (section 1.5.2) may serve in BAP-regulation of shoot meristem genes. This study is consistent with previously described cytokinin-induction of *ARR5* (Rashotte et al., 2003), a component

of two-component signalling in *Arabidopsis*. However, while cytokinins have been described as rapidly inducing *ARR5*, followed within two hours by lowered transcript levels (Brandstatter and Kieber, 1998), in the present study, *ARR5* transcript levels remained elevated at least 48 h after BAP treatment. As *ARR5* is expressed in developing leaves and shoot apical meristems (Che et al., 2002), this suggested that BAP-induced activity persisted in these tissues.

In the present study, BAP increased transcripts of *WUS* and *ARR7* (Figure 3.12 and Table 3.22). A relationship between two-component systems and regulation of meristem function was shown by an ethanol-inducible overexpression allele (Roslan et al., 2001) of *WUS* decreasing transcript levels of *ARR7* (Leibfried et al., 2005). The intricate relationships of *WUS*/*CLV3* and *WUS/AG* involving feedback loops of transcription suppression and induction may serve as models for the interplay between *WUS* and *ARR7*. Cytokinins may directly affect this relationship or coincidentally alter transcription of these genes by alternate means, such as the CLV pathway. The data here support recent assertions that the regulation of *WUS* is complex and not fully understood (Green et al., 2005).

Genes associated with cytokinesis were mined from the data, but none showed a significant response to BAP, 48 h after treatment (Table 3.8). Cytokinin induction of cytokinesis and stimulation of meristematic activity suppose a link between these processes (Mok and Mok, 2001). Increased transcript of D-type cyclins (*CycD3s*) is associated with expanding tissues and absent in differentiated organs (Dewitte et al., 2003). Genetic studies suggest cytokinin activation of *CycD3s* may be important to the phenomenon of cell division (Riou-Khamlichi et al., 1999). *CycD3* expression has been reported in association with proliferating shoot tissues in seedlings treated with BAP or zeatin; in contrast, *CycD3* transcript levels are not increased in *clv1*, a mutant featuring enlarged shoot and floral meristems (Riou-Khamlichi et al., 1999). The present study suggests that the extra floral organ phenotypes induced by BAP did not require cytokinin-induced *CycD3* expression.

4.1.2 Other Floral Phenotypes

BAP treatment induced several other aberrant floral phenotypes, including

ectopic bud formation and sepals bearing bract-like forked trichomes (Figure 3.10), characters resembling the *ap1* mutant (Gustafson-Brown et al., 1994), as previously described in studies of exogenous cytokinin and synthetic growth regulator activity (Venglat and Sawhney, 1994; 1996). By KDE® criteria, significantly altered *AP1* transcript levels were limited to the third BAP-treated replicate (Table 3.9). RT-PCR analysis was similarly inconclusive (Figure 3.18). As ectopic bud formation, resembling *ap1*, was a relatively rare phenotype in BAP-treated flowers (Table 3.4), it is likely that BAP-suppression of *AP1* transcript levels would also be limited; therefore, steady state levels of *AP1* transcript in most BAP-treated plants may have masked repressed levels in a few. Indirect evidence concerning *AP1* transcription was also sought. Decreased transcript levels of *SPL3* in BAP-treated populations (Table 3.9) suggested the possibility of cytokinins inducing *ap1* phenotypes through the action of *SPL* transcription factors. In *Antirrhinum*, *SQUAMOSA* is orthologous to *AP1* (Huijser et al., 1992) and *SPL* transcription factors are proposed to play a role in the regulation of *AP1* expression in *Arabidopsis* (Cardon et al., 1997).

BAP-treated flowers produced mosaic organs of petaloid-sepals, petaloid-stamens, and rare carpelloid-stamens (Figure 3.10). In untreated *Arabidopsis*, the initiation of four median long stamens is closely followed by the production of petal primordia (Smyth et al., 1990); therefore, in this study, it was not surprising that petaloid-stamen intermediate organs occurred more frequently in medial positions. Organs of sepaloid tissue with marginal petaloid tissue and petal-stamen mosaics in BAP-treated flowers resembled *ap1*, *ap2*, and *ap3* mutant floral phenotypes (Bowman et al., 1989; Bowman, 1993). In the BAP-treated samples of this study, no clear changes in transcript levels of genes responsible for chimeric organ phenotypes were observed. Previous studies have shown intermediate and strong transgenic lines of *LFY*::*WUS* and *AP3*::*WUS* produce carpelloid stamens (Lohmann et al., 2001). The occasional occurrence of these organs in BAP-treated plants may have reflected BAP-induced ectopic temporal and/or spatial expression of *WUS*. DNA-binding sites for *LFY* and *WUS*, have been identified in the second intron of *AG*, suggesting a regulatory role for *WUS* in flower development (Lohmann et al., 2001). Increased levels of *WUS* in BAP-treated samples suggested the potential for enhanced transcription of *AG*, a homeotic

gene regulating the development of stamens and carpels, although the phenotypic consequences of this situation were not detectable.

Arrested bud development was a common phenotype in the third BAP-treated replicate (Table 3.4, Figure 3.10). A preliminary hypothesis that this might represent a general stress response was not confirmed by GO analysis of the replicates; R3 did not target stress genes at a higher rate than the R1 and R2 (Figure 3.5). Alternatively, it was noted that the arrested bud phenotype in BAP-treated plants resembled the floral phenotype of *35S::NAP* transgenics, featuring short petal and stamen lengths and delayed deciduousness (Sablowski and Meyerowitz, 1998). Of the three BAP-treated replicates, R3 showed the greatest increase in *NAP* transcript abundance (Table 3.9).

Although a corresponding phenotype was not determined, BAP treatment induced increased transcript levels of the transcription factor *MYB13* (Table 3.26), a gene typically expressed in axillary buds and at the base of developing flowers (Kirik et al., 1998). This may represent a pathway for cytokinins to influence lateral shoot growth and inflorescence architecture. The regulatory region of *MYB13* includes the evening element motif, common in genes responding to light and/or entrained by the circadian clock, suggesting that this transcription factor may serve in cytokinin regulation of responses to light stimuli.

ANT-LIKE was the only other flower development gene mined from the microarrays that showed a significant increase in transcript levels. *AINTEGUMENTA* (*ANT*) serves in ovule development (Krizek et al., 2000); less is known about the regulatory role of *ANT-LIKE*.

4.1.3 Inheritance of BAP-induced Floral Phenotypes

Inheritance of BAP-induced floral phenotypes in T_1, T_2, and T_3 (Figure 3.22) suggested gene(s) serving in flower development might have been epigenetically altered. Not all of the treated plants responded to BAP, and even fewer showed inherited aberrant floral phenotypes; therefore, harvesting populations for microarray analysis likely diluted the transcript levels of those individuals possibly carrying epigenetic modifications. With this in mind, a less stringent significance threshold was applied to the T1 data (light blue and orange shading in tables represent a 2 to 2.5 fold change).

An increased floral organ number phenotype observed in T_1 populations (Table 3.33) suggested a gene serving in meristematic function might have been epigenetically modified in the treated population. *CLV1* had slightly lower transcript levels in the C2/T1 microarray sample than C1 control, although higher than BAP-treated samples (Table 3.6), suggesting the possibility of a potential BAP-induced epigenetic state for the T_1 chromatin. Interestingly, aberrant floral phenotypes in populations descended from BAP-treated plants were similar, i.e., resembled *clv* and *ap1*, to those found in plants with hypomethylated genomes (Jacobsen et al., 2000). Growing BAP-treated plants and T_1 offspring in cool temperatures, which increased the occurrence of aberrant flower development (Figure 3.23), suggests that BAP may have induced hypomethylation, as low temperatures can increase demethylation (Burn et al., 1993).

While *Arabidopsis* flowers naturally vary in the number of stamens, with one of the lateral stamens not developing in up to 25% of flowers (Smyth et al., 1990), a higher rate of a decreased-stamen phenotype was observed in T_1 plants than controls or BAP-treated populations. Since it is not known what factors influence the naturally occurring variation, it is speculative as to what factors were responsible for this heritable variation.

In the present study, *SUP* was flagged A(bsent) in all microarray samples, typical of many transcription factors. However, the phenotype of increased stamen number in BAP-treated populations, and decreased stamen number in subsequent generations, suggests BAP-induced heritable changes in expression levels of *SUP* should be investigated further. *SUP* functions in the regulation of stamen production: *sup* mutants feature stamens in whorl four (Bowman et al., 1992), and transgenic plants with ectopic *SUP* expression (through an *AP1* promoter) showed decreased stamen number in whorl three (Yun et al., 2002). In *ddm1*, a mutant with general hypomethylation, hypermethylation of *SUP* is observed, suggesting that some genetic sequences, such as *SUP*, are specifically targeted for methylation (Jacobsen et al., 2000; Kishimoto et al., 2001)Finnegan and Kovac 2000). Altered methylation patterns of the *SUP* locus, and upstream regions thereof, result in changes in expression of *SUP*, ultimately affecting stamen number (Bowman et al., 1992; Lindroth et al., 2001; Jackson et al., 2002b); hypermethylation silences *SUP* in the *clark kent* epimutant (Jacobsen and Meyerowitz, 1997). Similar to *SUP*, *AG* encodes a transcription factor functioning in flower

development that also shows hypermethylation propensity in hypomethylated *Arabidopsis* (Jacobsen et al., 2000), suggesting that this gene might also be a good candidate for additional studies of cytokinin-induced epigenetic inheritance.

4.2 Plant Responses to Environmental Conditions

Hormones play an important role in the integration of developmental processes with perceived environmental conditions (Hare et al., 1997; Bleecker, 1999). The microarrays of BAP-treated samples indicated a correlation between cytokinin regulation and environmental stimuli, in particular light and pathogen responses; the genes with the highest increase and decrease in transcript levels encode a light/auxin-responsive GH3 protein, *GH3-12*, and a pathogenesis-related protein (At4g33720), respectively (Tables 3.13, 3.14). Based on Meta Analyzer, BAP-effects on stress-associated genes are coincidental, or possibly coordinated, with regulation by other hormones, and also overlap with response to pathogens and ozone stress (Figure 3.19). BAP-induced changes implicated hormonal crosstalk and identified signal transduction components (section 3.5), which may be informative in establishing cytokinin-regulated pathways serving in responses to environmental factors.

4.2.1 Cytokinins and Light

The BAP-treated microarray data suggested that cytokinin regulation and transduction of light responses is intricate and complex. As previously discussed (section 4.1.1), cytokinins may integrate shoot proliferation with light responses through the regulation of shoot apical meristem function genes such as members of the *CLV1* pathway. Extrapolating from this evidence, coincidental BAP-effects on expression patterns of genes regulating shoot meristems and flower development and genes associated with light responses may indicate the evolution of cytokinin-induced mechanisms to promote shoot growth and rapid, prolific reproduction, in coordination with an exploitation of light resources.

As a side note, care must be taken in extrapolating any light-associated results reported in *Arabidopsis* to other species, as almost 60 years ago it was noted that members of the Brassicaceae are especially sensitive to blue light, a phenomenon nicknamed the ‘cruciferous quirk’ (Funke, 1948; Sage, 1992).

4.2.1.1 Cytokinins and Biological Clocks

Analysis of light-mediated processes in plants is complicated by the presence of at least two distinct modes of gene expression regulation. The timing of many light associated pathways is synchronized via entrainment by a central oscillator system consisting of at least one biological clock; light can also affect plant processes independent of clock function (Bünning, 1967; Kreps and Kay, 1997; McClung, 2006). Coordination of appropriate gene expression regulating physiological processes involves flexibility in the circadian clock. The planet rotation is fixed, while seasonal cycles include fluctuations in light and temperature. As a consequence circadian clocks maintain a 24 h period with flexibility in the phase of entrained rhythms; however, light and temperature cues and endogenous regulators are integral to the clock phase being synchronized to environmental conditions (Eriksson and Millar, 2003). BAP treatment may have altered the phase of a circadian clock, resulting in a cascade of transcript changes and accelerated flower timing in the next generation.

BAP affected transcript levels of genes central to clock function (Table 3.34) and those known to oscillate in a circadian rhythm (Tables 3.35, 3.37), in a manner suggesting that exogenous cytokinin treatment shifted the phase of the circadian clock in wild type *Arabidopsis*. This is supported by similar findings of Salomé et al. (2006) with cytokinin treatment of mutants. Genes with known oscillation patterns consistently showed BAP-altered transcript levels in R1, R3, and T1, but not R2. Although not foolproof, this pattern of altered transcript was used to recognize genes potentially responding to BAP via secondary regulation through altered clock phase. This was also useful as an indication of which genes experienced altered transcript levels in the next generation as a consequence of BAP adjustments to the clock.

As cytokinins are associated with responses to light cues, it is not surprising that they would also serve in plant biological clock function; however, to date, the role of cytokinins in the regulation of oscillating gene expression patterns has received relatively little attention (Thomas et al., 1997; Salomé et al., 2006).

BAP increased the transcript levels of type-A *ARR* element, *ARR4* (Table 3.22), which interacts with PHYB in a red light dependent manner (Sweere et al., 2001). Recently, Salomé et al. (2006) have shown that the double mutant *arr3,arr4* has a

lengthened circadian clock period, partially through disruption of the interaction of *ARR3* and *ARR4* with PHYB. The same study showed that exogenous cytokinins, in a dose dependent manner, affect the phase, but not the period, of clock-associated genes when applied to the *arr3,arr4* mutant (Salomé et al., 2006). In the present study, differences in transcript levels between wild type replicates treated with the same concentration of BAP suggested that variables other than dose are involved in determining direction and/or magnitude of the phase shift. Perhaps treatment timing is involved, analogous to animals, where the timing of exogenous melatonin affects phase entrainment (Rajaratnam and Redman, 2002).

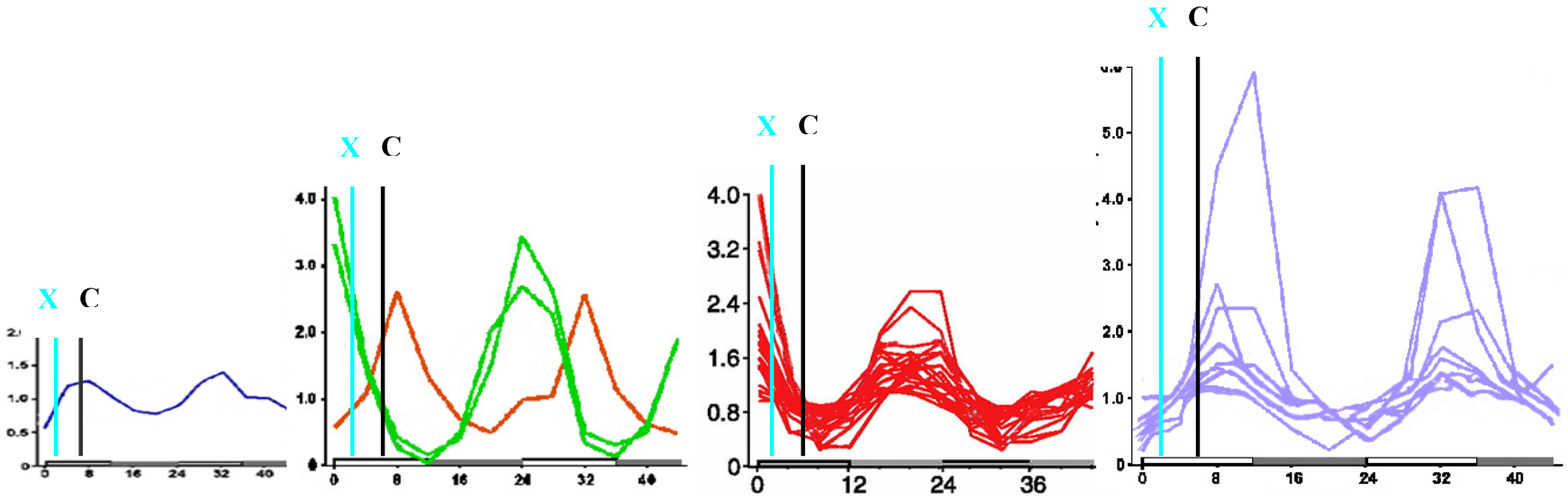
Beyond the preliminary findings of Salomé et al. (2006) that transgenic overexpression of type-A *ARRs* affects circadian function, other genes in the ARR family warrant attention as potentially mediating cytokinin zeitberg-function. The 30-plus *ARR* and *ARR-LIKE* genes in *Arabidopsis* are divided into three subgroups: type-B with a MYB-like domain, type-A lacking the domain (Imamura et al., 1998), and *pseudo-ARRs* (*APRRs*) with a phosphor-accepting receiver domain but lacking the phosphor-accepting Asp site found in both type-A and B ARRs (Makino et al., 2000). Type-B ARRs are described as positive regulators of cytokinin responsiveness and function in the induction of type-A *ARR* transcription (Hwang and Sheen, 2001; Kieber, 2002). *PCL1*, a gene sharing sequence identity with the DNA-binding domain of type-B ARRs, is required for increasing expression of *CCA1/LHY* (Hazen et al., 2005; Salomé et al., 2006). Although exogenous cytokinins do not directly affect transcript levels of type-B *ARRs* (Rashotte et al., 2003), *PCL1* transcript levels were lower in BAP-treated plants of the present study (Table 3.34), suggesting that a relationship between cytokinins and the role of *PCL1* in clock function should be investigated.

Another member of the *ARR* family, *APRR9*, showed increased transcript levels in BAP-treated samples (Table 3.22), although not in the heritable manner typical in this study of genes associated with the circadian clock. An evolutionary model suggests phytochromes, as pseudo-His kinases, may associate with *APRRs*, resembling a light-responsive two-component signal transduction system (Eriksson and Millar, 2003; Mizuno and Nakamichi, 2005). A quintet of *APRR* genes, *APRR9,7,5,3,1*, serve intricate, if not essential, roles in controlling the period of circadian rhythms (Ito et al.,

2003; Nakamichi et al., 2005). *APPR9/7/5* activates *APRR1* (*TOC1*), as part of a loop at the center of *Arabidopsis* circadian clock (Mizuno and Nakamichi, 2005). *APRR1*, induces the synthesis of the photosynthetic apparatus and is a central component of the biological clock in *Arabidopsis* (Somers et al., 1998a; Mizuno and Nakamichi, 2005). That BAP treatment increased transcript levels of *APRR9* differs from a previous study reporting that cytokinins do not affect transcript levels in *APRRs* (Rashotte et al., 2003). This might reflect differences in experimental procedures, for example, the impact of BAP on *APRR9*, whether or not directly as part of the function of cytokinins as zeitbergs, may take up to 48 h.

Opposing changes in transcript levels of *CCA1*/*LHY* and *PCL1* in BAP-treated samples (Table 3.34) supports the notion that BAP induces a shift in the phase of the circadian clock, as the action of *CCA1/LHY* serves in the negative arm of the circadian clock, resulting in a 12 h cycle of inverse expression with *PCL1* (Alabadi et al., 2001; Hazen et al., 2005). While most clock genes maintained steady state transcript levels in R2, *CCA1/LHY* showed lower levels, as opposed to higher levels in R1 and R3 (Table 3.34), suggesting that these genes may be important components of cytokinin regulation. *APRR1* (*TOC1*), *PCL1*, *GI*, *COR15b*, *COR413*, *KIN1*, and *KIN2* showed varying degrees of response to BAP (Tables 3.34, 3.35, 3.38), most often with the strongest response in R1, R3, and T1 samples. These genes have been described with oscillating expression patterns (Harmer et al., 2000; Hazen et al., 2005) and regulatory regions upstream of the genes include the evening element (AAAATATCT) promoter motif, which is integral to biological clock regulation by the two highly conserved MYB-related transcription factors *CCA1* and *LHY* (Wang et al., 1997; Schaffer et al., 1998; Harmer et al., 2000; Alabadi et al., 2001). The evening element promoter element was also found in an unknown light responsive gene responding to BAP in the proposed circadian pattern, At4g04330 (Table 3.35), warranting further investigation of entrainment of this gene.

Speculation of details of the BAP-effect on the circadian clock phase was based on comparisons of the present transcriptomic data with known oscillation patterns of specific genes. For example, *COR15b* and *KIN2* transcripts cycle in a 20-28 h



Y-axis represents relative intensity of transcript levels (ratio) X-axis represents a time course (h)

Figure 4.1. Genes with oscillating expression patterns, modified from Harmer et al. (2000). "0" denotes subjective sunrise. "C" denotes approximate collection time of T_1 populations. "X" denotes relative transcript levels indicated by microarray data. *PHOT1* (blue); *CCA1/LHY I* (green); *GI* (orange); phenylpropanoid biosynthesis genes (red); stress genes including *COR15b* and *KIN2* (lilac). It appears that BAP treatment altered the phase of the circadian clock in a heritable manner by approximately 3-5 h.

oscillation, peaking 8 to 10 h after sunrise and *CCA1*/*LHY* has its lowest level 12 hours after sunrise (Harmer et al., 2000). Relative transcript abundance for these genes in control and BAP-treated samples indicated that BAP treatment probably induced a lagging shift of 3 to 5 h in the clock phase (Figure 4.1). In contrast, BAP induction of a leading phase shift in R2 might account for the reduced transcript levels of *CCA1*, *LHY*, and *TT6*, although it would not account for those genes that maintained steady state transcript levels in R2. Over- or under-expression of key genes in clock function can eliminate circadian oscillation (Alabadi et al., 2001; Más et al., 2003), so it is possible that BAP treatment may have induced arrhythmicity in R2. However, the resulting non-oscillating transcript levels are unlikely to have aligned with oscillating patterns in controls.

The T1 microarray data showed increased transcript levels of *CCA1/ LHY*. Hypocotyl elongation has been reported in plants over-expressing these genes (Schaffer et al., 1998; Wang and Tobin, 1998); however, this phenotype was not observed in offspring of BAP-treated plants. This was interpreted as further evidence that the increased transcript levels of *CCA1/LHY* represented a shift in the phase of the biological clock rather than a stable increase in gene expression. It is also possible that potential hypocotyl elongation was offset by other inherited effects from the exogenous cytokinin, as this treatment can induce de-etiolation, mimicking the light response of dark-grown seedlings (Chory, 1994; Su and Howell, 1995), as discussed later (section 4.2.1.3).

The BAP-induced changes in genes associated with circadian rhythm suggested that cytokinins might be added to the current model of clock function (Figure 4.2). The proposed effect of BAP on clock regulation suggests that genes associated with cytokinin signal transduction might be emphasized in clock processes. It has been reported that cytokinins can activate gene expression via transcription factors *ARR1* and *ARR2* binding to the cis-element 5'-AGATT-3' (Sakai et al., 2000; Sakai et al., 2001; Rashotte et al., 2003). As this sequence is a reverse complement of the *CCA1* binding evening element, 5'-AA(A/T)ATCT-3' (Harmer et al., 2000), a link between cytokinin regulation, two-component systems, and the circadian clock is further supported and should continue to be investigated.

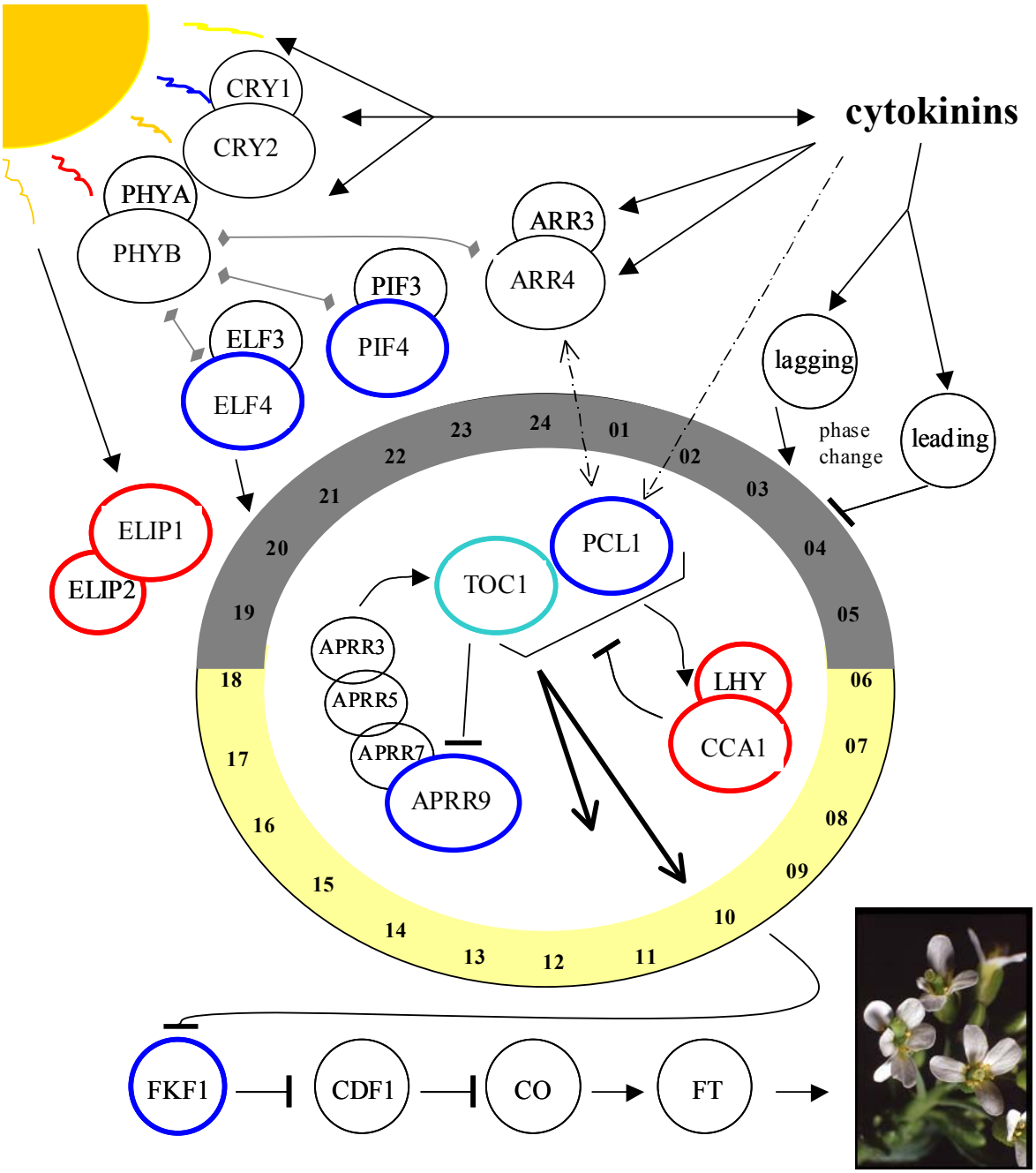


Figure 4.2. Circadian clock system and long day flowering in *Arabidopsis*. Modified from Eriksson and Millar (2003) and Nelson et al. (2000). Central to the clock, *TOC1* and *PCL1* activate *CCA1/LHY*, which decay at the end of the day. Light-activated *APRR9* begins a cascade affecting *TOC1*. PHYB binds with ARR4, PIF4, and ELF4. *ARR4* functions in light effects within and/or independently of relationships with PHYB and cytokinins. *PCL1* has sequence similarity with type-B ARR genes, and therefore may interact with cytokinins and type-A ARR genes (e.g., *ARR4*). The clock regulates *FKF1* expression, which rises during the day; FKF1 degrades CDF1, a negative regulator of *CO*. Long days increase *CO* and promote flowering. ELIP1&2 provide protection from UV damage. Circle colour indicates T1 data: red increased transcript levels, blue decreased, light blue slightly decreased. T_1 populations flowered earlier.

The coincident role of plant photoreceptors and cytokinins in regulating circadian rhythms is of interest. Potentially, PHYs directly regulate transcription responses of clock elements; the biologically active form of PHYB in red light responses interacts with PIF3 and PIF4, which mediate resetting of circadian clock oscillation through upregulation of *CCA1*/*LHY* transcription (Ni et al., 1998; Yanovsky and Kay, 2001; Huq and Quail, 2002). CRYs, the blue light receptors in animals and plants, have proven to be integral components of central clock regulation in mammals (van der Horst et al., 1999); however, it is still not clear how these photoreceptors serve in entraining the endogenous oscillator to the light cycles of the environment (Somers et al., 1998b).

Endogenous cytokinin concentrations, measured independently in shoot and root tissues of carrot, were found to oscillate in a 24 h period (Stiebeling and Neuman, 1986). A similar cycle was observed in *Populus robusta* where the changes were interpreted as reflecting light intensity (Hewett and Wareing, 1973). In both cases, cytokinin concentrations peaked between 1 and 2 p.m. approximately, similar to the timing of the highest levels of *PHY* and *CRY* activity. The promoter activity of *CRY2* is predominate in the root meristem and shoot primordia (Toth et al., 2001), important sites of cytokinin metabolism and action, respectively. Speculatively, these spatially and temporally coordinated cycles may serve to optimize regulatory roles of cytokinins in concert with photoreceptors.

4.2.1.2 Cytokinins and Flowering

That T_1 plants flowered earlier than controls by ontogenetic age, i.e. number of rosette leaves at anthesis was decreased by 19%, suggested flower timing can be accelerated by cytokinins in a heritable fashion. A BAP-altered flowering phenotype would not be expected in the present study, since the plants were treated after vegetative to inflorescent meristem conversion. Miller (1956) observed similarities between cytokinin-responses and red light effects and suggested the two may act through the same biological mechanism. He recommended that this warranted more thorough testing, in particular with regards to identifying whether cytokinins affect flowering in the same manner as red light.

The early flowering phenotype observed in the T_1 generation, in the present study, may have been connected to BAP-altered transcript levels of *CCA1*, *LHY*, *ELF4*, *FKF1*, and *GI* (Table 3.34), genes associated with long day photoperiod transition to flowering, within, or independent of, alterations to the circadian clock (Rédei, 1962; Levy and Dean, 1998; Piñeiro and Coupland, 1998; Fowler et al., 1999; Nelson et al., 2000; Doyle et al., 2002). The effects of BAP on other flower timing genes were inconclusive, especially as many were flagged A(bsent), including *CO*, *FLOWERING LOCUS T* (*FT*), *TERMINAL FLOWER1* (*TFL1*), *FLOWERING LOCUS C* (*FLC*), and *FRIGIDA* (*FRI*).

Lowered transcript levels of *FKF1* may be indicative of BAP-altered function of a flower-timing pathway functioning in conjunction with the circadian clock (Figure 4.2). Entrained in a circadian rhythm, FKF1 targets proteins for ubiquitination and has an essential role in the degradation of CYCLING DOF FACTOR 1 (CDF1), a suppressor of *CO* (Nelson et al., 2000; Imaizumi et al., 2005) (Figure 4.2). Also within circadian clock regulation, *GI* plays a role in flower induction by increasing *CO* and *FT* transcript abundance (Mizoguchi et al., 2005). Localized in the nucleus, GI seems to serve in PHYB signalling (Huq et al., 2000) and may regulate circadian clock function (Park et al., 1999). As light and temperature are both important regulators of temporal responses, it is also of note that *GI* responds to low temperatures with a 5 to 8-fold increase in transcript (Fowler and Thomashow, 2002). BAP treatment was found to decrease transcript levels of *GI* in the T_1 population, suggesting that cytokinins may affect *GI* transcript levels as part of a light rather than temperature response.

4.2.1.3 Cytokinins and Seedling Development

Cytokinins have been reported as localized to chloroplasts (Swaminathan et al., 1977), where they are thought to serve in the induction of chloroplast development and in sustaining chlorophyll levels as an aspect of delaying senescence. Possibly as an aspect of regulating these processes, the present study showed that BAP treatment lowered transcript levels of *PORA* (Table 3.36), a gene integral to chloroplast biosynthesis in seedlings. Functioning specifically in etioplast differentiation during seedling development, *PORA* transcript levels decrease shortly after etiolated seedlings

are illuminated (Runge et al., 1996; Kim et al., 2005). *PORA* contains the circadian-associated evening-element in its regulatory region (Harmer et al., 2000); however, the BAP-altered pattern here also included higher transcript in R2, suggesting that cytokinin regulation of *PORA* might occur outside of clock entrainment, perhaps indicating a role for cytokinins in the greening of young plants upon soil emergence.

The 13-fold increase in transcript levels of *ELIP1* in BAP-treated plants (Table 3.36) may be an aspect of cytokinin roles in seedling maturation and/or limiting photo-damage. Serving in chlorophyll accumulation in the seedling response to light signals *ELIP1* and *ELIP2* are nuclear genes encoding thylakoid membrane proteins (Kloppstech, 1985; Grimm and Kloppstech, 1987; Casazza et al., 2005). BAP also increased transcript levels of *STH* (Table 3.35) a zinc finger transcription factor associated with promoting *HY5* activity, serving in de-etiolation of seedlings in light (Osterlund et al., 2000). The microarray pattern suggests the circadian clock entrains *STH*.

4.2.1.4 Cytokinins and Light Stress

While plants are dependent on light as an energy source, stress is also integral to this relationship. As an aspect of, or independent of, affecting the phase of the circadian clock, the present data showed BAP altered the transcript levels of genes associated with offsetting light stress. Early in chloroplast development unabsorbed photons can be toxic to immature cells. Specific stress-inducible pathways, including the biosynthesis of photo-protective pigments such as carotenoids and xanthophylls, are thought to have evolved to offset potential light damage (Harari-Steinberg et al., 2001). These plant flavonoids, a diverse family of aromatic compounds providing organ colouration includes chalcones, flavones, and anthocyanins, are instrumental in protection against UV light (Winkel-Shirley, 2001).

BAP increased transcript levels of components of flavonoid biosynthetic pathways (Table 3.37) including *CHS*, which catalyzes the first committed step in flavonoid production, and *TT* genes. Specific and diverse roles have been described for the *TT* genes responding to BAP in the present study: *TT3* is integral to flavonoid biosynthesis (Winkel-Shirley, 2001), *TT6* contributes to UV protection (Li et al., 1993b), *TT19* functions in the transport of flavonoids to the vacuole (Kitamura et al., 2004), and

TT7 encodes a P450 cytochrome that is as a membrane anchor for the flavonoid enzyme complex (Hrazdina and Wagner, 1985; Schoenbohm et al., 2000).

The increased levels could be an aspect of early observations of flavonoid biosynthesis that suggested light and cytokinin induction are additive or slightly synergistic (Koehler, 1972). Transcriptomic studies of light and exogenous BA have shown that cytokinins only invoke anthocyanin accumulation in the presence of light but do not affect transcript levels of *CHS* when the response is saturated in light (Deikman and Hammer, 1995).

Most of these genes of flavonoid biosynthetic pathways, showed increased transcript abundance in the BAP-treated samples in the R1, R3 and C2/T1, but not R2, the pattern interpreted as indication of entrainment by the circadian clock. The increased transcript levels of some anthocyanin-related genes in the T_1 generation of this study likely reflected a heritable phase-shift of a clock entraining anthocyanin biosynthesis; however, specificity in the regulation of flavonoid pathway elements was suggested by the data, as the transcript levels of *PAL1* and *TT5* did not respond in a similar pattern. Deikman and Hammer (1995) suggested that *PAL1* and *TT5* are post-transcriptionally controlled by cytokinins. The decreased transcript levels of *TT6* in R2 of the BAP-treated samples is of note and may indicate that this gene has an upstream position in the relationship between cytokinins, circadian clocks, and flavonoid biosynthesis.

The connection between cytokinins and anthocyanins may also include the GST family, which functions in a variety of stress responses, including light signalling (Loyall et al., 2000) and members of which had altered transcript levels in BAP-treated samples (Table 3.17). Specifically, anthocyanins, which are produced in the cytoplasm, must eventually be localized in the vacuole. GSTs, together with cytochrome P450s and the glutathione pump, serve in tagging toxic substances, including secondary metabolites such as anthocyanins, and transporting them to the vacuole before they endanger the cells producing them (Marrs, 1996). BAP treatment induced transcript levels of genes essential to anthocyanin biosynthesis and containment may be aspects of the hormone's regulation of light-stress responses.

BAP treatment also increased transcript levels of *ELIP1,*2 (Table 3.36). These genes are described with a photoprotective function based on the *Arabidopsis chaos*

mutant, which is unable to accumulate ELIPs during light stress and suffers photo-oxidative damage when exposed to high light and chilling conditions (Hutin et al., 2003). The naturally high cytokinin concentrations occurring in early afternoons (Hewett and Wareing, 1973; Stiebeling and Neuman, 1986) would correspond with a potential high light stress, which might benefit from cytokinin-induction of protective measures.

Phenotypically, anthocyanin biosynthesis and accumulation was suggested by a purple tinge in rosette leaves of BAP-treated plants. While these phenotypes were not observed in T_1 plants, *CHS*, integral to anthocyanin production, was upregulated by BAP in the treated parental population, and increased transcript levels were also found in the next generation. Methylation-pattern regulation of *CHS* transcription has previously been observed in petunia (Jorgensen, 1995), suggesting a potential mechanism of epigenetic inheritance.

BAP treatment also increased transcript levels of two members of the *Arabidopsis GH3* family (Table 3.13). Genes of this family, upregulated by exogenous auxin in plants grown in white light (Tanaka et al., 2002), are also induced by far red light treatment, which mimics end of day conditions or a shade stress (Smith and Whitelam, 1997). The especially strong induction of *GH3-12* by BAP four or more days after treatment suggests a convergent point between cytokinins, auxin, and light-responsive pathways.

4.2.1.5 Cytokinins and Light/Temperature Responses

Previous work has reported a link between light and low temperature responses in plants (Krapp and Stitt, 1995; Strand et al., 1997; Fowler and Thomashow, 2002; Blázquez et al., 2003). Although cold acclimation is obviously temperature-regulated, freezing tolerance can also be induced by high-light, as demonstrated by the induction of *COR* genes by each stimulus (Dexter, 1933; Gray et al., 1997). Contrarily, BAP-lowered transcript levels of several *COR* genes, some in the pattern suggesting secondary regulation through BAP-altered circadian clock entrainment (Table 3.38). Oscillating expression of *COR15b* and *KIN2* has been previously reported in *Arabidopsis* (Harmer et al., 2000) and the results here suggest that studies of light alteration of *COR* gene

transcript levels should be interpreted with altered circadian clock and/or cytokinin levels in mind.

Meta Analyzer indicated that ethylene lowers transcript levels of *COR* genes (Figure 3.25), suggesting the BAP effect on these genes may have been secondary through induction of elements of the ethylene biosynthetic pathway (Table 3.20). Crosstalk in the regulation of *COR* genes within a diurnal rhythm is suggested by reports of coincidental daytime high levels of cytokinin and ethylene levels, in carrot and *Sorghum*, respectively (Stiebeling and Neuman, 1986; Finlayson et al., 1998).

In an apparent paradox to the BAP-repression of *COR* gene transcript abundance observed in the present study, exogenous cytokinins have been shown to increase resistance to cold damage in cucumber, tomato, and corn crops (Budykina et al., 2000; Zauralov et al., 2000). The ability of exogenous cytokinins to induce cold tolerance may be species-specific or may reflect genes outside the *COR* gene regulon. For example, the BAP treatment of *Arabidopsis* here indicated cold tolerance might have been affected through non-*COR* cold response genes, such as *RHL41* (Table 3.13). Absence of transcriptome data from the previous studies prevents comparisons of cytokinin-effects on gene expression in the different species.

RHL41, encoding a zinc finger protein, serves as a convergent point of light response and cold acclimation. Overexpression of *RHL41* induces light-acclimation symptoms, including increased chlorophyll content, thicker palisade mesophyll, and increased anthocyanin production (Iida et al., 2000). Transcript levels of *RHL41* have also been described as entrained by the circadian clock (Fowler et al., 2005); however, in this study a significant increase in *RHL41* transcript levels in BAP-treated samples was not found in the next generation, unlike other clock-entrained genes affected by BAP. This suggested that *RHL41* might have been directly induced by BAP as part of a stress response, rather than indirectly through a phase-shift of the biological clock. *WRKY25*, which acts downstream of *RHL41*, is elevated in response to oxidative stress associated with high light conditions (Rizhsky et al., 2004), and BAP-treated samples showed significant increases in *WRKY25* transcript levels. This suggests a regulatory role for cytokinins within a light-induced oxidative stress pathway.

4.2.2 Cytokinins and Defense Responses

The high fitness and energy costs of disease resistance and stress responses are partially controlled by the repression of associated regulatory pathways until a sufficient threshold of stress is reached. Much remains to be determined about the hormonal regulation and molecular mechanisms of these processes and the present study indicates cytokinins play an integral role. BAP treatment induced numerous stress response genes, in particular ones associated with light stress, as discussed (section 4.2.1.2), and disease resistance (Tables 3.14, 3.15). Similar to previous reports of exogenous cytokinins indirectly regulating gene expression via induction of ethylene biosynthesis (Yip and Yang, 1986), BAP increased transcript levels of elements of the ethylene biosynthetic pathway (Table 3.20). Ethylene serves as an intermediate signalling agent in the regulation of numerous defense responses to environmental stimuli, including pathogens, ozone, temperature, drought, and wounding (Schaller and Kieber, 2002). The genes associated with defense responses with increased transcripts in this study (Table 3.14) showed coincidental upregulation by pathogens and ozone in Meta Analyzer (Figure 3.19). BAP induction of ethylene may have been a factor as defense against the pollutant ozone and biotic elicitors have coincidental pathways mediated by ethylene (Sandermann et al., 1998).

Predominant stresses endured by plants include high light stress, temperature extremes, drought, herbivory, and pathogen attack. When the stimuli are functionally related, such as heat and drought inducing desiccation, convergence of responses can be expected. In contrast, when opposing conditions occur, hormone regulation must prioritize responses. The microarray data of this study indicated that BAP synchronously induced genes associated with light stimuli and pathogen defense (Figure 3.19). A connection between these seemingly disparate environmental cues might be expected from an evolutionary perspective, as shoot damage in response to high light, and associated drought stress, would leave a plant vulnerable to pathogens.

BAP treatment increased transcript levels of *PR1,2,5* (Table 3.14). In *Arabidopsis*, light-sensitive mutants exhibit a light-dependent increase in salicylic acid levels and a consequent increase in *PR1*, a gene associated with pathogen resistance. The convergence of response pathways is supported by mutants deficient in phytochrome

proteins having repressed *PR* gene expression and lowered resistance to pathogenesis (Genoud et al., 1998). Serving in numerous stress responses, the BAP treatment increased transcript levels of fourteen *GSTs* and decreased one, *GSTF14* (naming convention Wagner et al. 2000). The best characterized of the *Arabidopsis GSTs*, *GSTF6* is induced by multiple factors, including UV light, ozone, exogenous ethylene, auxin, and salicylic acid (Levine et al., 1994; Horvath and Chua, 1996). *GSTF6* is commonly co-induced with *PR1*, so has become a marker for stress responses. Coincident increases in transcript levels of *PR1* and *GSTF6* in the BAP-treated samples is interpreted as a component of cytokinin regulation of pathogen responses. Although little is known about the regulation and functions of most individual members of the GST family, the differential regulation of *GSTF14* might be connected to it having a unique amino acid grouping relative to others in its class (Wagner et al., 2002). That BAP affected specific *GSTs* supports the model of independent regulation of the GST family members (Wagner et al., 2002) and suggests a specificity for cytokinins in utilizing GSTs, possibly as part of responses to light stimuli and pathogens.

Convergence of pathogen defense and light response is also found in the production of some flavonoids, which serve as antimicrobial agents and herbivore deterrents, as well as providing solar protection (Jenkins et al., 2001; Wade et al., 2001; Winkel-Shirley, 2001) (section 4.1). The data here suggest cytokinins may play a role in regulation of these convergent defense responses. A link between cytokinins and pathogen resistance in the present data is supported by the report of tomatoes grown on fields fertilized by vetch-facilitated nitrogen fixation where increased cytokinin levels were associated with increased shoot growth and significantly increased disease resistance (Kumar et al., 2004).

Other genes associated with pathogen defense response processes, such as cell wall modifications, also responded to BAP treatment (Table 3.16). Of particular note, *PDF1.2* (Table 3.39) was a rare case of BAP-treated samples showing decreased transcript levels, while the T_1 generation showed increased levels. *PDF1.2*, an ethylene/jasmonic acid-dependent pathway marker gene functioning in cell wall modifications as part of disease defense (Penninckx et al., 1998), may have been epigenetically induced by BAP to increase pathogen resistance in the next generation.

A common component of pathogen defense is the isolation of infected tissues by PCD. Exogenous cytokinins have been found to induce PCD in a dose-dependent manner in carrot and *Arabidopsis*: low levels of BA (5 μM), did not induce PCD, while increased levels (13-27 μM) did (Carimi et al., 2004; 2005). In the present study, PCD was probably induced by the protocol of treating *Arabidopsis* with high concentrations (10^{-3} M) BAP. Leaf necrosis ensued quickly after BAP treatment, supporting this assumption. The associated organ-browning may have been indicative of an early stage of PCD, where chloroplasts are the first organelles targeted for breakdown (Lohman et al., 1994). Watering and covering the plants reduced the occurrence of tan-coloured leaves and plant death, following BAP treatment, consistent with previous reports that attenuating rapid spread of a hypersensitive response of PCD can be achieved by high humidity (Hammond-Kosack et al., 1996). Plants that survived the initial shock of BAP treatment produced robust shoot systems and remained green longer than controls, suggesting growth was not repressed by a stress response.

Besides a role in pathogenesis, it is possible that BAP induced an increase in transcript levels as an artefact of the treatment. Compounds that are normally produced by an organism can be regarded as xenobiotics when they occur at higher concentrations than usual. As *GST* expression is activated by pathogen attack and xenobiotics, the increased transcript levels of at least some of *GSTs* in the present study may have been in response to exogenous BAP being perceived as a xenobiotic compound, and an attempt to sequester it in the vacuole.

The evolution of complex plant immune systems that include feedback loops to recognize and resist microbial pathogens (Chisholm et al., 2006) offers a potential origin of the role(s) of cytokinins in pathogenesis. Shortly after the discovery of cytokinins, it was speculated that pathogens initiating gall tumours utilized production of this hormone to initiate rapid cell division (Braun, 1958; Thimann and Sachs, 1966). In the present study, the transcriptomic data indicated high cytokinin levels induced genes associated with pathogenic resistance. As cytokinin production is increased by a number of pathogens, including bacteria, e.g., *Rhizobium*, fungi, e.g., *Helminthosporium* Link ex Fries, and slime mould, e.g., *Dictyostelium* (Morris, 1986), it is speculated here that high cytokinin concentrations may serve as a trigger of a plant's pathogen defense

mechanisms. Cytokinins, at levels generated by pathogen-initiated plant cell factories, which the exogenous BAP of this study may have mimicked, might serve as specific elicitors of stress responses, subsequently initiating ethylene and salicylic acid biosynthesis, cell wall modifications, and GST activity.

It has been proposed that kinase systems evolved in single-celled organisms to sense and respond to external conditions, and then diversified their function in multi-cellular lineages to coordinate growth through cell to cell communication (Clark et al., 1997). With a role in shoot meristem function, *CLV1*, a LRR receptor kinase, is structurally similar to receptor kinases that function in pathogen recognition and resistance, for example, *Cf-9* in tomato and *Xa21* in rice (Jones et al., 1994; Song et al., 1995; Clark et al., 1997; Martin, 1999). Furthering the analogy between the *CLV1* pathway and pathogen responses, a large family of CLAVATA3/ESR-related proteins, functioning as activation ligands to receptor kinases, such as CLV1 (Cock and McCormick, 2001), share sequence similarity with an esophageal gland cell protein from *Heterodera glycines*, a soybean cyst nematode (Olsen and Skriver, 2003). Meta Analyzer indicates *CLV1* expression is repressed by biotic stresses. In the present study, BAP treatment lowered transcript levels of *CLV1* and it is possible that cytokinins evolved the capacity to regulate and integrate development and pathogen resistance, through *CLV1*, and similar, pathways.

4.2.2.1 Cytokinins and Ethylene

The function of *ACS5*, encoding a member of the initial step in ethylene biosynthesis, is affected by auxin and cytokinins in etiolated *Arabidopsis* seedlings: auxin increases transcription of *ACS5*, while cytokinins increase the stability of the protein via translational modifications (Vogel et al., 1998; Chae et al., 2003). The present study found that BAP significantly elevated the transcript level of *ACS2*, a member of a family of genes encoding factors in the rate-limiting step of ethylene biosynthesis, suggesting the capacity of cytokinins to also affect ethylene production at the level of transcription. Light regulation of oscillating ethylene levels in relation to functioning PHYB has been documented (Finlayson et al., 1998), and the data here suggest that cytokinins should be investigated as an intermediate factor.

It has been suggested that ethylene signaling systems evolved from the adaptation of early eubacteria-like His receptor kinases and eukaryotic signal transduction MPK cascades into potential modules for crosstalk with other regulatory pathways featuring similar components (Bleecker, 1999; Bleecker and Kende, 2000). The present data suggest that the *MPKs ZIK8* and *MPK11* (Table 3.24) should be investigated for a role in crosstalk between cytokinin and ethylene.

4.2.2.2 Cytokinins and Senescence

Cytokinins function in the regulation of processes delaying senescence (Schenk et al., 2005). It would seem contradictory for cytokinins to function in delaying aging and inducing pathogen resistance, as the latter has been tied to accelerated senescence (Barth et al., 2004); however, it was not clear if the senescence, light, and pathogen responses regulated by BAP acted synergistically, antagonistically, or independently. Independent regulatory functions for cytokinins in these processes might be based on localization or concentration of the hormone.

BAP treatment lowered transcript levels of genes Meta Analyzer indicated as having increased levels in senescent tissues, including *P5CS1* (Table 3.38) and a senescence-like gene (At2g21045) (Table 3.11). Genes downregulated in senescent tissues were induced by BAP, e.g., *TCH3, TIR, EDS1, HSP81-1,* and *PAD3,4* (Table 3.14). These genes might be investigated further for their role in the cytokinin-induced delay in aging processes, especially *P5CS1*, which BAP affected in a heritable manner.

In the present study, BAP treatment decreased transcript levels of *SEN1* in R3 (Table 3.11). A microarray database indicates lowered transcript levels of *SEN1* in response to zeatin; whereas, it serves as marker for both plant defence and senescence responses (Quirino et al., 2000; Schenk et al., 2005). A pathway for cytokinin-regulation of *SEN1* was implied by BAP induction of *WRKY6* (Table 3.27), a transcription factor known to repress *SEN1* (Robatzek and Somssich, 2002). These data indicate the plants recognized BAP as a cytokinin and the treatment invoked appropriate genetic processes to reduce natural aging processes. The altered transcript levels of *SEN1* in the T_1 population (Table 3.35), initially seemed relevant to inheritance of cytokinin regulation of aging; however, altered transcript of this gene, an important marker of integration of

pathogenesis and senescence, may have been the result of it being gated by the circadian clock (Schenk et al., 2005).

YLS9 had significantly increased transcript levels in BAP-treated tissues and Meta Analyzer indicated that it also responds to zeatin with increased transcript levels. YLS9 is localized to the chloroplast and is associated with pathogen-induced senescence, independent of salicylic acid signalling (Zheng et al., 2004). Although not measured, perhaps increased or localized endogenous zeatin levels in BAP-treated plants regulated this pathway.

Cytokinins generally serve as positive growth regulators, responding to favourable environmental conditions by increasing shoot growth and delaying senescence (Richmond and Lang, 1957; Mok, 1994) and the BAP treatment in the present study delayed aging in mature plants by several days. However, the role of cytokinins in delaying senescence has been challenged by transgenic *Arabidopsis* transformed with increased expression of CKX, resulting in lowered endogenous cytokinins; these plants unexpectedly showed delayed aging processes (Eckardt, 2003; Werner et al., 2003). As CKXs degrade zeatin, and have little affect on BAP-type cytokinins (Brzobohaty et al., 1994), the present data suggest that naturally occurring BAP-type cytokinins may play an important role in the regulation of aging processes.

4.2.3 Cytokinins and Co-regulation of Significant Genes

Groups of two to five genes shared coincidental BAP-altered transcript levels, biological function, and physical proximity (Table 3.29). Although genes involved in developmental processes and responses to environmental conditions are usually scattered throughout the genome, examples of functionally-related, co-expressed genes clustered on chromosomes have been recognized in the sequenced genomes of humans (Lercher et al., 2002), yeast (Cohen et al., 2000), and plants (Zhao and Last, 1996). An underlying factor in this phenomenon, especially in angiosperms, is extensive gene and chromosomal segment duplication (Gachon et al., 2005). For example, sharing 95% sequence identity suggests *KIN1&2* represent a duplication event (Zhizhong Gong et al., 2001). As might be expected, many cases of coordinated gene expression represent parallel tandem repeats of genes; however a substantial number of co-expressed genes

are non-homologous (Zhan et al., 2005). As well as tandem genes on the same DNA strand, coordinated expression occurs in pairs of genes on opposite strands, where transcription may converge or diverge.

The light responsive kinases sharing altered transcript levels in BAP-treated samples and chromosomal proximity have divergent transcription. Genes with divergent transcription have a higher statistical occurrence of co-expression than convergent or tandem, perhaps due to close physical proximity of promoter sites (Cohen et al., 2000).

Promoter motifs common to several clusters of genes identified in the BAP-treated data as co-regulated suggest a propensity to defense response, with possible regulatory crosstalk involving cytokinins, ethylene, salicylic acid, and jasmonic acid. Evolutionarily it would be expected that co-regulated genes might utilize transcription factor binding motifs to maintain coordinated expression in association with shared functional specialization (Lynch and Conery, 2000).

4.3 Epigenetic Inheritance

Epigenetic inheritance has been defined as meiotically heritable gene expression that involves chromatin modification, without altering the DNA sequence (Wu and Morris, 2001). Altered floral phenotypes in the T_1 generation of BAP-treated plants (section 3.6.1) were the first indication of epigenetic inheritance in this system. The transcriptomic data analysis (section 3.6.3), which compared coincidental patterns of transcript abundance in controls, BAP-treated populations, and non-treated progeny, supported that epigenetic inheritance was induced by BAP treatment in *Arabidopsis*. Speculation of the mechanisms of cytokinin-induced epigenetic inheritance follows.

Cytokinins, as modified adenines with the potential for incorporation into nucleic acids, have been implicated as possible DNA mutagens (Skoog, 1994). In the present study, inheritance of aberrant floral phenotypes and gene expression patterns adds to the speculation that cytokinins are able to alter the DNA sequence; however, the ephemeral state of BAP-induced altered floral phenotypes within the raceme and in subsequent generations suggested that at least some of the altered chromatin states, as opposed to mutated DNA, were both heritable and reversible. Also, the specificity of altered gene expression suggested sustained chromatin remodelling was involved rather than random

mutagenesis. DNA methylation, histone modifications, siRNAs, and RNA-mediated DNA methylation are mechanisms proposed to have the capacity to maintain specific gene expression patterns across generations (Gendrel and Colot, 2005); however, speculation on the role of cytokinins in chromatin remodelling is limited by our incomplete understanding of the processes responsible for establishing and maintaining gene expression patterns (Hashida et al., 2006). Reversibility of altered gene expression patterns is of general interest as it may have implications in transgenic technologies.

In the BAP-treated populations, more genes showed a significant increase in transcript levels (91%) than decrease (9%). The opposite was found in genes of the C2/T1 array; of the 247 genes with a 2.5-fold or greater change in transcript levels, 25% were increased and 75% decreased (Table 3.1). This was interpreted as evidence of a propensity for epigenetic inheritance of gene silencing, possibly involving DNA methylation, a mechanism of epigenetic modifications involving the addition or removal of a methyl group to select cytosine bases.

While methylation is a normal aspect of ontongeny in mammals and plants, genome reprogramming in sperm, eggs, and early embryos was generally thought to erase induced methylation patterns (Reik et al., 2001). Exceptions of specific inherited methylation patterns have been documented in plants, such as pollen development in *Nicotiana* (Oakeley et al., 1997), vernalization in *Triticum* (Sherman and Talbert, 2002), and flowering and floral development in *Arabidopsis* (Finnegan et al., 1996; Jacobsen and Meyerowitz, 1997; Kinoshita et al., 2004). The capacity for de novo altered methylation patterns in *Arabidopsis* is supported by the presence of an active DNA methyltransferase system (Doerfler, 1995).

The BAP-induced epigenetic inheritance proposed to have occurred in the present study, may have been the result of specific de novo methylation/demethylation patterns or flaws in the mechanisms designed to reset the chromatin state during DNA reproduction. Mutant analyses have identified *DEMETER* (*DME*) and *REPRESSOR OF SILENCING 1* (*ROS1*) as functioning in demethylation (Morales-Ruiz et al., 2006). In the present study, general demethylation was not indicated, as genetic and phenotypic changes associated with lowered methylation, such as a general increase in transcription, curly leaves, and *ap2-like* floral phenotypes as observed in *clf* (Finnegan et al., 1996),

were not found in BAP-treated plants. Contrary to the alteration of genome-wide methylation levels, the data of BAP-induced inheritance suggested the capacity of cytokinins to specifically target gene expression patterns linked to developmental processes and responses to environmental conditions; in particular, processes involved in flower initiation and development, light and temperature responses, and pathogen-defense responses. Much remains to be determined about the processes involved in establishing and maintaining DNA methylation patterns in plants, especially in progeny.

Inheritance of an extra floral organ phenotype suggested that chromatin remodelling might have affected the shoot meristem genes *CLV1* and/or *WUS*. A coincidence of endogenous zeatin distribution and increased DNA methylation in the central zone of the shoot apical meristem in *Prunus persica* (L.) Batsch. (Bitonti et al., 2002) supports speculation, in the present study, of the capacity of BAP to induce epigenetic inheritance in *Arabidopsis* through de novo methylation patterns. As discussed earlier (section 4.1), altered DNA methylation states may also have been a factor in the inheritance of other BAP-induced aberrant floral phenotypes.

One of the first documented cytokinin-binding affinities was to a protein complex in tobacco that functions as a S-adenosyl-L-homocysteine (SAH) hydrolase (Mitsui et al., 1993; Mitsui et al., 1996). SAH hydrolase catalyses the reversible cleavage of SAH into adenosine and homocysteine and, thereby, competitively inhibits DNA methylation by sequestering methyl groups from S-adenosylmethionine (SAMeth)-dependent methyl-transfer reactions (Rammesmayer et al., 1995). Mitsui et al. (1996) proposed that in tobacco, cytokinins affect DNA methylation/demethylation patterns through regulation of SAH hydrolase activity and HOMOLOGY-DEPENDENT GENE SILENCING 1 (HOG1), a factor in maintenance of gene silencing. In the BAP-treated samples, transcript levels of *HOG1* were not affected, but two genes encoding proteins with activity dependent on the transfer of a methyl group by SAMeth had significantly higher transcript levels (Table 3.41). The lowered transcript levels in the T_1 transcriptome may be an indication of cytokinin-binding restricting SAH activity, resulting in increased SAMeth mediation of methylation events, potentially leading to induced epigenetic inheritance of repressed gene expression.

The microarray data did not indicate a BAP-effect on the transcript levels of genes associated with chromatin remodelling (Table 3.41); however, this does not eliminate the possibility of these factors serving in the induction of epigenetic modifications earlier or later than the 48-hour time point of the microarrays. Also, as acetylation, methylation, phosphorylation, and ubiquitination are post-translational modifications to histones (Pandey et al., 2002), increased transcription rates of the various epigenetic machinery components need not be integral to a protein's role in chromatin remodelling, and, therefore, a transcriptomic study may not readily reveal BAP-induced changes in these processes.

Chromatin Assembly Factor-1 (CAF-1), a conserved histone chaperon complex supporting nucleosome assembly is composed of subunits encoded by *FAS1*, *FAS2*, and *MSI1*, in *Arabidopsis* (Kaya et al., 2001). The BAP-treated plants of this study shared phenotypes with *fas1* and *fas2* mutants, including increased floral organ number. In the mutants this phenotype is attributed to an expanded *WUS* expression domain (Kaya et al., 2001; Reyes et al., 2002). Altered expression of *SCARECROW* and reduced root elongation are found in *fas1* and *fas2* (Kaya et al., 2001). A member of the *SCR* transcription factor (Table 3.10) showed increased levels and roots appeared shorter in BAP-treated plants. Also functioning in chromatin remodelling is HAT1 (Vlachonasios et al., 2003), specifically regulating floral meristem activity through the *WUS/AG* pathway (Bertrand et al., 2003). A GCN5/HAT complex has been linked to the regulation of *COR* genes via altering acetylation patterns of histones interacting with *CBF1* (Stockinger et al., 1997). Although the transcript levels of *FAS1*, *FAS2*, and *HAT1* did not change in response to BAP-treatment (Table 3.41), a study of the potential relationship between cytokinins and CAF-l and other pathways affecting histone function is warranted based on a coincidental relationship with *WUS* and evidence here of the inheritance of BAP-altered meristem function.

4.3.1 Epigenetic Inheritance and Evolution

Approximately a decade ago epigenetic inheritance was implicated as evolutionarily significant when it was shown that methylation differences between maternal and paternal alleles of the mouse H19 gene were inherited (Tremblay et al.,

1995). The present study implicates the capacity of cytokinins to induce epigenetic inheritance, leading to altered phenotypes in floral development, precocious flower timing, and increased plant stature, across generations. This appears to be the first proposition of hormone-induced epigenetic inheritance in plants.

Cytokinins are regulators of shoot development, and also serve in responses to environmental conditions, including light stimuli and pathogen defense. A timekeeper role in fine-tuning biological clock entrainment appears to also fall under the regulatory repertoire of cytokinins. Epigenetic inheritance of cytokinin-induced regulation of development and responses to the environment, both through clock function and altered chromatin states of individual genes, could confer an advantage to offspring relegated to similar environmental conditions.

4.3.1.1 Epigenetic Model - Waddington's landscape

Published by Waddington in the 1950's, his model landscape captures the complexity of genetic control of developmental plasticity (Figure 4.3), and also could represent epigenetic inheritance of gene expression patterns. The surface contours represent the potential of an organism, the ball, at any point in time in life's journey, to be influenced by the spatial and temporal expression of its genetic blueprint, under the influence of environmental conditions. The underlying foundation of pegs represents the genetic alleles, and the wires the 'chemical tendencies of the genes'. These guy wires link the relatively stable genomic grid to the plasticity of the phenotypic landscape and are responsible for the dynamic state of the surface contours. The present study indicated that cytokinins have the capacity to modify specific genes in a heritable manner. Thus, hormone-regulated epigenetics can pre-establish plant responses to environmental conditions and developmental pathways established by the parents, in the offspring. Waddington's classic model of plasticity in development might include heritable states of gene expression patterns in the form of preset elastic cords rather than cables.

4.4 Microarray Experiment Design

The microarray is an efficient tool for studying transcript abundance linked to gene expression changes in response to specific factors; however, the relatively high cost

of GeneChips® and sample processing can restrict experimental design. Cooperative science alleviates this limitation through shared microarray databases, such as Genevestigator, although there are risks in over-interpreting data generated from varied protocols. Statistical interpretation of microarray data is an ongoing challenge for biological researchers (e.g., Quackenbush, 2002). Numerous computer programs have been developed for microarray data analysis; however, the complexity of the task requires a mixture of statistical validity and intuitive interpretations.

The microarray data of the present study showed that BAP affected transcript levels of genes serving diverse functions in plant processes. From the initially disparate evidence of BAP-induced changes in transcript abundance of individual genes, subsequent analyses led to interpretation of cytokinin-effects on the genetics of shoot meristem function, flower development, response to light and temperature, biological clock function, pathogenesis, signal transduction pathways, transcription regulation, and epigenetic inheritance. Any study of this nature is limited to the collective knowledge of the time; although sequenced, the functional roles of many genes in the *Arabidopsis* genome have yet to be established. Some genes encoding proteins of 'unknown' function were documented in the results of this study in anticipation of their characterization.

4.4.1 Timing of Tissue Harvest

While many transcriptomic studies harvest tissues within minutes or hours of implementation of experimental conditions, a 48 h post BAP-treatment collection time was chosen here for several reasons. First, the high rate of aberrant flower phenotypes induced by BAP in the first five flower positions (production of 1.9 flowers/day (Smyth et al., 1990)) suggested BAP-induced changes in gene expression persisted for at least 48 h. Second, the mechanisms for suppression of gene expression can take longer than induction, for example, 68% of gene repression in response to low temperatures takes 24 hours or longer to occur (Fowler and Thomashow, 2002). Third, it was reasoned that the delay might decrease gene expression reflecting a shock-reaction to the exogenous treatment, facilitating a more focussed examination of cytokinin-effects; cytokinins rapidly influence transcript abundance but also affect long-term changes in expression

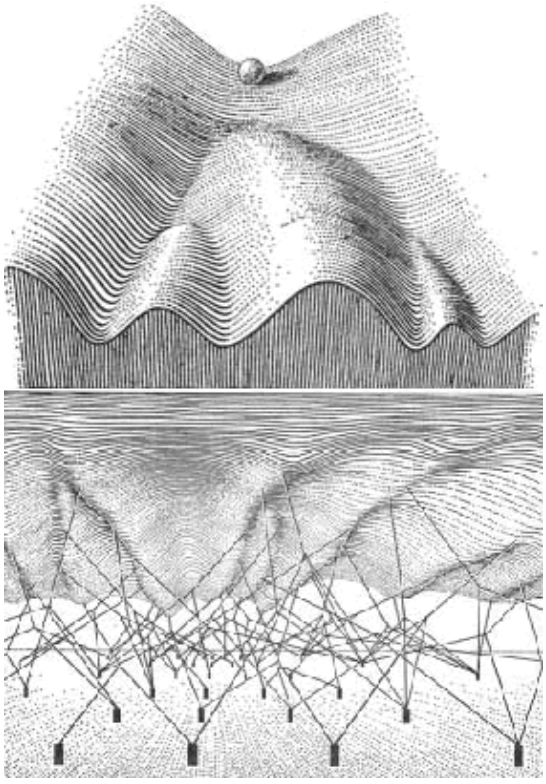


Figure 4.3. Waddington's "Epigenetic Landscape" (Waddington, 1957). Ball represents an organism (or organ) on a developmental pathway featuring plasticity. Signal transduction pathways below combine pegs representing genetic alleles and guy wires serving as gene expression patterns.

(Schmülling et al., 1997). Finally, epigenetic inheritance was indicated by aberrant floral phenotypes carried through to the next generation; it was hypothesized that BAP-induced changes in gene expression that persisted 48 h were more likely to be indicative of potentially heritable gene expression patterns.

4.4.2 Variability between BAP-treated Replicates

As discussed at length by Kreps et al. (2002), interpreting transcriptomic data requires caution, for microarrays undoubtedly include background variation, i.e., responses to subtle differences in experimental conditions. Statistically significant gene expression patterns induced by BAP here were supported by biological and technical replicates. However, disparities between the BAP-treated replicates R1-3 suggested background variation might have been a factor. Inconsistencies in gene expression between the replicates may have reflected several variables. First, imprecision is intrinsic to exogenous treatments. For example, contact of the concentrated BAP droplet with the plant surface could affect the rate and location of absorption; in this study, some droplets spread over petioles and/or lamina of cotyledons and leaves, while others formed a tight dome over the central region of the shoot meristem.

Examination of floral phenotypes and RT-PCR of gene expression in multiple BAP-treated populations, in the present study, also argued that exogenous experiments could not be precisely reproduced. For example, forked trichomes on sepals were common but inconsistent on BAP-treated flowers. More dramatic, some plants featured trichomes arising on pistils, while most did not. Some phenotypes were common within, but rare between, populations. For example, arrested bud development – flowers persisting as diminutive buds, with tiny petals slightly protruding from the sepals, on the rachis as the internodes elongated, without anthesis or organ abscission – occurred in 13% of the flowers in the first five flower positions of R3 but was not observed in populations harvested for R1 or R2. There was no apparent experimental protocol discrepancy to explain the generally higher rate of variation and occurrence of aberrant floral phenotypes in R3, which also showed the highest number of genes with a significant change in transcript levels.

The response to exogenous hormones can be sensitive to the developmental stage at the time of treatment (Venglat and Sawhney, 1996). While consistency in treatment time was attempted in this study, it was difficult to determine subtle differences in development. An attempt was made to treat the plants shortly after the transition from vegetative to inflorescence function, a protocol resulting in diverse aberrant floral phenotypes (Venglat and Sawhney, 1996). However, precision in BAP application was hindered by the induction of flowering not being visible to the naked eye. Floral primordia initiation at the time of treatment was observed at the shoot apex with SEM, and dissection microscopy also confirmed bud initiation. However, the time factor and organ damage associated with microscopy prevented observations of the numerous individual plants treated with BAP in populations from which the RNA for microarrays and RT-PCR was extracted. The SEM-documented plants were, by necessity, a sub-population of the treated populations and were not included in the final RNA. Therefore, rather than precision based on primordia initiation, vegetative features were used to determine treatment timing: two cotyledons, 2 leaves with a lateral orientation, and 2-3 visible emerging leaf primordia (termed here the 4-5 leaf stage).

Complicating interpretation of exogenous cytokinin-effects is a positive correlation between BA/BAP concentration, the production of endogenous isoprenoid-type cytokinins, and the induction of cytokinin oxidases (Vanková et al., 1987; Auer et al., 1999). The microarray data here indicated systems regulating endogenous cytokinin metabolism responded to the application of BAP. *Arabidopsis* encodes a small family of seven cytokinin oxidases, of which *CKX3*,*4*, and*5* showed significant increase in transcript levels (Table 3.18). *CKXs* degrade excess cytokinins by cleaving N6 side chains, releasing adenine (Chatfield and Armstrong, 1986). However, that *CKXs* have been shown to be limited in their ability to cleave side chains in BA-style cytokinins (Brzobohaty et al., 1994; Mok and Mok, 2001) offers an explanation for the capacity of exogenous BAP to affect gene expression patterns 48 h and more after application and throughout the initiation of over 10 flower primordia.

In this study, BAP treatment may have induced increased levels of zeatin-type cytokinins, which have been shown to be elevated by exogenous BAP in tobacco tissue culture systems (Hansen et al., 1987); however, zeatin levels were not measured. BAP

induction of zeatin, in concert with upregulation of genes encoding tissue-specific zeatin-degrading compounds (*CKXs*) (Werner et al., 2003), could alter the spatial distribution of cytokinin ratios, with affect regulatory consequences.

Besides variation in timing of treatment and endogenous responses affecting cytokinin levels and distribution, the conditions of harvesting tissue for RNA extraction may have influenced the results. Although not suspected at the time of experimental design, circadian variation in transcript abundance was subsequently interpreted as an important factor in the effects of BAP on the transcriptome. Therefore, timing of tissue collection should have been better synchronized.

Independent of computer analysis, it was noted that several genes responded to BAP in R1, R3, and C2/T1, while transcript levels in R2 maintained steady state or changed in the opposite direction (e.g., Tables 3.34-3.38). The lack of response in R2 may have reflected disparity in experimental methodology as speculated above or, for example, there may have been subtle differences in conditions, such as unaccounted light, mechanical stimulation, or unknown variations.

Validity of statistical analyses is contingent on experiment design and program parameters. In the present study, the three biological replicates (R1-3) of BAP treatment were compared to two controls. The use of T_1 populations as the second control, C2, no doubt resulted in putative false negatives in statistical analysis by SAM®. Also, a 2.5-fold threshold of significance was implemented, which, although common in the literature, is an arbitrary value. In the present study, the mRNA samples represented a large population of plants, not all of which displayed altered phenotypes. Therefore, those not responding to BAP may have diluted transcript levels of the respondent plants, resulting in false negatives. Lowering the significance threshold is argued for specific cases, such as transcription factors, as low-level changes in these catalytic components of expression pathways can be biologically significant.

With due recognition of these variables, consequences, and limitations, there was ample evidence that the experiments of the present study successfully examined phenotypic and transcriptomic effects of cytokinins on plant biology and, therefore, may contribute to our present understanding.

5. CONCLUSIONS

Plant growth and differentiation are continuously altered and adjusted by hormone regulation of responses to a milieu of stimuli. This study addressed cytokinin regulation of the *Arabidopsis* transcriptome and subsequent phenotypic outcomes. BAP affected transcript abundance of genes responsible for shoot meristem activity, flower development, light-associated processes, two-component systems, cytokinin metabolism, auxin regulation, ethylene and salicylic acid biosynthesis, receptor kinases, Ca^{2+} signalling, and transcription factors. The BAP-treated transcriptomic data correlated with publicly available microarray databases revealed complex direct and indirect regulation of responses linked to environmental stimuli, especially light and pathogen attack. The present study also identified the capacity of cytokinins to induce specific heritable gene expression patterns, at least in part by altered entrainment of the circadian clock, and implied directed epigenetic chromatin remodelling targeting specific genes and pathways.

1. Numerous studies have documented phenotypic changes induced by exogenous hormones, and microarray analyses have offered overviews of gene expression, but rarely have the two been combined. Transcriptomic data integrated with mutant or transgenic phenotypes supported previous hypotheses that cytokinins alter flower morphology by affecting transcript levels of key genes with shoot meristem function, especially *CLV1* and *WUS*. The capacity of WUS to repress transcriptional activity suggests it may have played an integral role in BAP-induced epigenetic inheritance. BAP-induced phenotypes, comparable to those of known floral mutants and transgenic plants, were coincident with altered transcript levels of genes functioning in associated pathways. Rapid restoration of wild type floral development in the raceme of treated plants and subsequent generations is consistent with the supposition that basic reproductive morphology is highly buffered and stabilized by canalization.

2. An important aspect of hormonal regulation, that is, the ability to sense stimuli and coordinate an appropriate response, is a task largely accomplished by networked kinase receptors and transcription factors. Responses to cytokinins, long recognized as mimicking physiological reactions to red light, were found in BAP-altered transcript abundances of numerous genes associated with light cues. The effect of BAP on light-associated genes associated with auxin regulation, such as members of the *GH3* family, indicates pathways warranting future investigation to further our understanding of crosstalk between these important hormones in responses to the environment. BAP altered the expression of several signal transduction pathway components, however, the role of cytokinin in regulating many of these pathways is not clear at this stage.

3. BAP, as an elicitor or regulator, invoked stress response genes, apparently in concert with salicylic acid and ethylene. It is speculated here that high cytokinin levels might have evolved as an immune response elicitor, based on evolutionary relationships with cytokinin-producing pathogens, including specific bacteria, fungi, or slime mould. As cytokinins appear to influence disease resistance in a heritable manner, potential agricultural applications might be investigated.

4. The measure of hormone-induced inheritance observed in the present study suggested cytokinins might serve as a zeitgeber, or timekeeper, in regulating changes induced by light in setting or re-setting biological clocks. Cytokinin-altered transcript levels in the subsequent untreated generation emphasized genes associated with light-responses, especially those with oscillating expression patterns entrained by biological clocks, such as components of anthocyanin biosynthesis and light-sensitive, cold-inducible *COR* genes. Further work is required to determine potential correlations between endogenous cytokinin levels, variable light conditions, and circadian gene expression. Experiments involving altered cytokinin levels should be diligent in assessing the potential impact on biological clock entrainment, to minimize misinterpretation of resulting gene expression data.

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